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EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) is a refereed, quarterly international journal financed by the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University, Jordan. JJBS celebrated its 12th commencement this past January, 2020. JJBS was founded in 2008 to create a peer-reviewed journal that publishes high-quality research articles, reviews and short communications on novel and innovative aspects of a wide variety of biological sciences such as cell biology, developmental biology, structural biology, microbiology, entomology, molecular biology, biochemistry, medical biotechnology, biodiversity, ecology, marine biology, plant and animal biology, plant and animal physiology, genomics and bioinformatics.

We have watched the growth and success of JJBS over the years. JJBS has published 14 volumes, 60 issues and 800 articles. JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Clarivate Analytics- Zoological Record and recently has been included in the UGC India approved journals. JJBS Cite Score has improved from 0.7 in 2019 to 1.4 in 2021 (Last updated on 6 March, 2022) and with Scimago Institution Ranking (SJR) 0.22 (Q3) in 2021.

A group of highly valuable scholars have agreed to serve on the editorial board and this places JJBS in a position of most authoritative on biological sciences. I am honored to have six eminent associate editors from various countries. I am also delighted with our group of international advisory board members coming from 15 countries worldwide for their continuous support of JJBS. With our editorial board's cumulative experience in various fields of biological sciences, this journal brings a substantial representation of biological sciences in different disciplines. Without the service and dedication of our editorial; associate editorial and international advisory board members, JJBS would have never existed.

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Finally, JJBS would not have succeeded without the collaboration of authors and referees. Their work is greatly appreciated. Furthermore, my thanks are also extended to The Hashemite University and the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research for their continuous financial and administrative support to JJBS.

Professor Atoum, Manar F.
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The Evaluation of Secondary Metabolites in *Saccharum officinarum* L. and *Mimosa invisa* Mart. as Natural Herbicides

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Abstract

The giant sensitive plant (*Mimosa invisa* Mart.) is a major weed found in the sugarcane (*Saccharum officinarum* L.) farming areas, which dominates and adversely affects the quantity and quality of the harvest. This weed poses a threat to both the sugarcane farmers and sugar companies because it causes about a 6 % to 9 % decline in the plant's biomass and a 0.09 % reduction in the crop yield. *M. invisa* has great competitiveness against the crop plant *S. officinarum* and also an increased population efficiency of 14.08 % in the first year (plant cane) to 38.55 % in the third year (3rd ratoon). Therefore, this research aims to determine the metabolic compounds in the rhizosphere of *M. invisa* and *S. officinarum* and assess their roles as allelochemicals. Research was conducted at the Laboratory of Agrotechnology, Faculty of Agriculture and Animal Science, University of Muhamadiyah Malang, and Central Laboratory of the Indonesian Legume and Tuber Crops Research Institute, Malang. The field research was carried out in the Research Garden Krebet Baru Sugar Factory, Malang, East Java, Indonesia. The descriptive qualitative research design was used and data were arranged randomly in groups of three treatments and three replications. The treatment details were planted in different plots of land each as, T_1: *Mimosa invisa* only, T_2: *Mimosa invi*sa mixed *Saccharum officinarum,* and T_3: *Saccharum officinarum* only. Furthermore, the analysis of metabolic compounds, using the Gass Cromatografy Mass Spectromotometry (GCMS), showed the presence of octadecanoic acid (an allelochemical compound) and methyl ester (2.06 %) in the rhizosphere of *M. invisa* and *S. officinarum*. These metabolite compounds are commonly used as herbicide activators.

Keywords: Allelochemical, Bio-herbicide, Eco-friendly technology, Giant sensitive plant, Sugarcane.

1. Introduction

According to the Central Statistics Agency (*Badan Pusat Statistik*) in 2019, the required consumption of sugar in Indonesia reached 5.1×10^6 t, while national sugar production is currently 2.36×10^6 t. In addition, there was a decline in national sugar production which was up to 2.22×10^6 t (Cindy, 2020). This decrease in sugar production was a result of the raw material, i.e. sugarcane (*Saccharum officinarum* L.), facing challenges such as competition with weeds which has, in turn, impacted the yield of sugarcane production. This weed poses a threat to both the sugarcane farmers and sugar companies because it causes about a 6 % to 9 % decline in the plant's biomass and a 0.09 % reduction in the crop yield. Additionally, the presence of this weed during the vegetative growth stage of the sugarcane crops results in a yield reduction of up to 40 %. Conversely, the weed competition at the growth stages of 3 wk, 6 wk, and 9 wk after planting the crop resulted in a decrease of 77.6 %, 50.6 %, and 4.7 % in the yield (Concenço *et al*., 2016; Faheem and Muhammad, 2015; Fanny *et al*., 2019; Gulshan and Bhagirath, 2020; Peng 2012; Sugar Research Australia, 2021).

In this stuation, the giant sensitive plant — *Mimosa invisa* Mart (also referred to in the literature as *Mimosa diplotrica*, C Wright) is a field crop weed that causes a decrease in the quality and quantity of crop production (Dania and Bamidele, 2006; Osariyekemwen, 2020; Prajal *et al*., 2020; Rao *et al*., 2018). It has been shown in previous research that *M. invisa* has great competitiveness against the crop plant *S. officinarum* and also an increased population efficiency of 14.08 % in the first year (plant cane) to 38.55 % in the third year (3rd ratoon) (Aekrathok, *et al.* 2021; Jayasree, 2006; Phil and Emilie, 2017; Zainol, 2017). Additionally, the mechanism of competition

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between weeds and cultivated plants growing around them is through allelopathy, whereby biochemicals released by the weeds inhibit the growth of other plants around them (Abu-Romman *et al*., 2015; Chauhan, 2008; Dania and Bamidele, 2006; Gulshan and Bhagirath 2020). Conversely, allelopathy occurs when a chemical component produced by plants interacts with other plants growing around them. It is used by weeds to compete with other plants growing around them (*Aliyu et al,* 2018; Ejaz, 2003; Erida *et al*. 2019). Subsequently, the results obtained will reveal that *M. invisa* weed extract can inhibit the germination of seeds of other plants growing in its vicinity, giving it the potential of a bioherbicide (Albuquerque *et al.,* 2011; Ejaz, 2003; Hussain *et al*., 2021; Permatasari *et al*., 2020; Sadiqullah *et al*., 2019). Therefore, this research aims to determine the metabolite compounds in the giant sensitive plant (*M. invisa*) weed rhizosphere and sugarcane plant (*S. officinarum*) together with their roles as allelochemicals.

2. Materials and Methods

2.1. Field Research

This research was conducted in the upland (non-irrigated) area of the Research Garden Farming Facilities (*Bina Sarana Tani*) Krebet Baru Sugar Factory, Bululawang, Malang, East Java, Indonesia (coordinant 120° 37' 30" to 70° 58' 10") in Latosol soil, pH 5.5. Additionally, the climate classification of Schmidt and Ferguson was included in type C (slightly wet) grading. The average monthly rainfall data for the last 10 yr also showed that it amounted to 1 898.2 mm, with rainfall of about 102.5 d.

The plant *S. officinarum* was planted in a double row system using the stem cutting BL (Bululawang) cultivar, with a center-to-center spacing of 100 cm to 110 cm and a width of 50 cm to 60 cm. The fertilizers NPK were used at the rates of 150 (N), 105 (P_2O_5), and 150 Kg (K_2O), respectively. The BL stem cutting was also planted in October during the beginning of the rainy season.

Consequently, the seeds of *M. invisa* were taken from the sugarcane cultivated fields and sown in polybags. These weeds were then maintained up to a height of approximately 10 cm. Furthermore, after the sugarcane seeds had sprouted, these weeds were planted with them according to the treatment.

The descriptive qualitative design was used for this research, and the experimental units were arranged randomly in groups consisting of three treatments and three replications. T_1 plot of land was planted with *M. invisa* only, T_2 with *M. invi*sa and *S. officinarum*, and T_3 with *S. officinarum* only. The data was analyzed using the standard deviation with the Equation (1) (Adinurani 2016 ; Iftikhar and Hayat, 2021)

$$s = \sqrt{\frac{n\sum_{i=n}^{n} x_i^2 - (\sum_{i=1}^{n} x_1)^2}{n(n-1)}} \quad (1)$$

Notes: S = standard deviation ; X_i = value of X to i ; n = sample size

2.2. Laboratory Test

This research was conducted in the Laboratory of Agrotechnology, Faculty of Agriculture and Animal Science, University of Muhamadiyah Malang, and the Central Laboratory of the Indonesian Legume and Tuber Crops Research Institute, Malang, East Java, Indonesia within February to May 2019. Furthermore, the metabolite compound was analyzed using a Gas Chromatography-Mass Spectrophotometry (GC-MS) type QP2010S Shimadzu (Japan), semi-polar column RXi-5MS, with Helium carrier gas flow rate of 0.5 mL min^{-1}, and pressure of 27.4 kPa. The initial temperature of the GC 120 ^oC oven was increased with a speed of 5 ^oC min^{-1} until it reached 320 ^oC min^{-1} and the sample volume of 1 uL to 2 uL (Ahmed and Annadurai, 2017). Also, the data obtained were analyzed with the NCBI (National Center for Biotechnology Information) database to obtain specific metabolites.

2.3. Roots Extract of Saccharum officinarum and Mimosa invisa

The roots of *S. officinarum* and *M. invisa* weeds were extracted using the reference-based method (Gusthinnadura, *et al*. 2017; Kokosa, *et al*., 2019; Ram, *et al*. 2019). Conversely, the roots of both plants were taken 15 cm to 20 cm from the base of the rootstock in a circle as deep as 25 cm to 30 cm, to ensure they remained intact without damage. After collection, these roots were washed with water and extracted as follows; 10 g of the roots sample were crushed by Bamix, and 1:2.5 mL of absolute methanol solvent was added. The Rotator shaker gemmy-VRN-360 was then used as an extractor for 2 h. Afterward, 5 mL supernatant was added at an absolute methanol ratio of 1:1, after which the extract underwent centrifugation using the Centrifuge Digital DLABDM 0412 at a speed of 4 000 rpm for 15 min (1 rpm = 1/60 Hz).

Furthermore, 7.5 mL each of supernatant and cold methanol was added in the ratio 1:1. This also underwent centrifugation using Centrifuge Digital DLABDM 0412 at a speed of 4 000 rpm for 15 min. Lastly, the pellets were taken and 1 mL absolute methanol was added in preparation for GC-MS testing which was done at a retention time of 39.46 min.

3. Results and Discussion

3.1. Results

The results of the analysis of four rhizosphere samples from *M. invisa* and *S. officinarum* plants showed the presence of many types of allelopathic compounds, also known as allelochemical compounds. These different compounds are identified as shown in the following, Figure 1, Figure 2, and Figure 3.

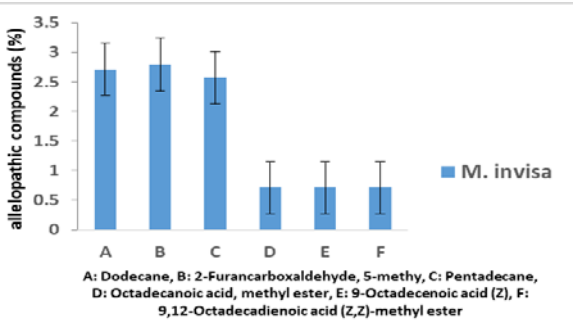

Figure 1. Allelochemical compounds from rhizosphere samples of *M. invisa*

From Figure 1 above, the results showed that the *M. invisa* root extracts from the rhizosphere when grown alone contained allelochemical compounds such as Dodecane (6.83 %), 2-Furan carboxaldehyde, 5-methyl (7.28 %), and Pentadecane (6.10 %).

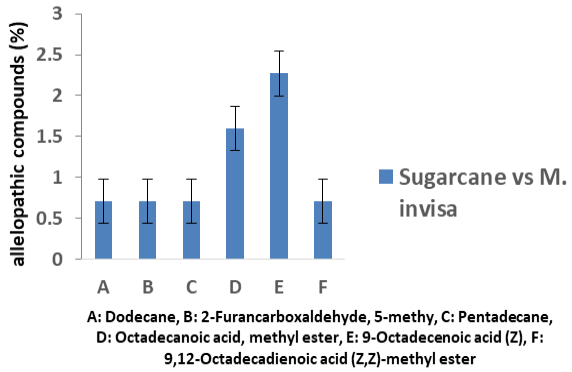

Figure 2. Allelochemical compounds from rhizosphere samples of *S.officinarum* mixed with *M. invisa*.

Figure 2 above showed both *S. officinarum* and *M. invisa* in the rhizosphere when grown side by side contained Octadecanoic acid, methyl ester (2.06 %), and 9,12-Octadecadienoic acid (Z, Z)-methyl ester (3.86 %).

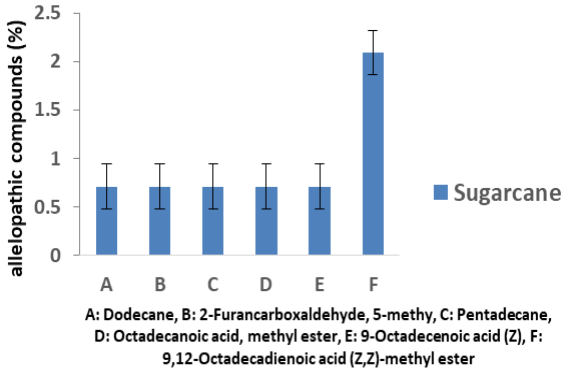

Figure 3. Allelochemical compounds from rhizosphere samples of *S. officinarum*

The Figure 3 above shows that the results of the identification of *S. officinarum* root extract from the rhizosphere when grown alone contained allelochemical compounds such as 9-Octadecenoic acid (Z) 4.64 %.

The results of the GC-MS chromatogram of dodecane compounds are presented in Figure 4.

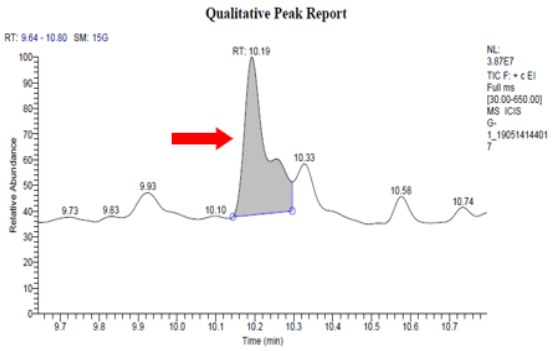

Figure 4. The GC-MS chromatogram of dodecane compounds.

Figure 4 above showed that the rhizosphere of the weeds contained allelopathic compounds such as dodecane (6.83 %). Additionally, dodecane is a metabolite compound in plants that has the molecular formula $C_{12}H_{26}$ with a molecular weight of 170.33 g mol^{-1}. This compound is classified as essential oil which has the chemical name dihexil. Furthermore, dihexil is used in micro-extraction solvents for the formation of the herbicide, Triazine, which is an active herbicide used to control both grassy and broadleaf weeds. (Kokosa, 2019).

The results of the GC-MS chromatogram for the compound 2-furan carboxaldehyde, 5-methyl are presented in Figure 5.

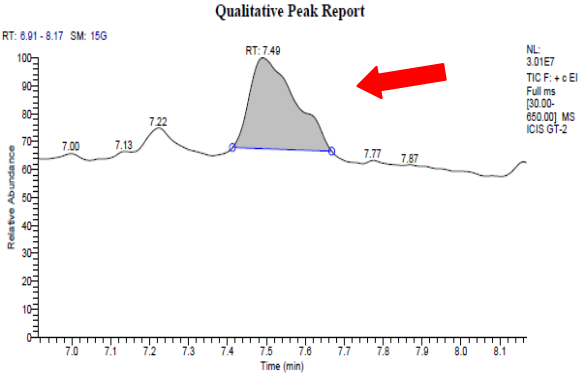

Figure 5. The GC-MS chromatogram for the compound 2-furan carboxaldehyde, 5 methyl.

Figure 5 above shows the results of the GC-MS chromatogram of 2-furan carboxaldehyde, 5-methyl compounds. Conversely, the compound 2-furan carboxaldehyde, 5-methyl, with the molecular formula, $C_6H_6O_2$ are found in weed root extracts and belong to a class of furfural compounds that have allelopathic potential in plants. In addition, it is also one of the main allelochemical compounds in the methanol root extract from the marigold plant (Genus: *Tegetes* L.) (Chotsaeng, 2018).

The results of the GC-MS chromatogram of pentadecane compounds are presented in Figure 6.

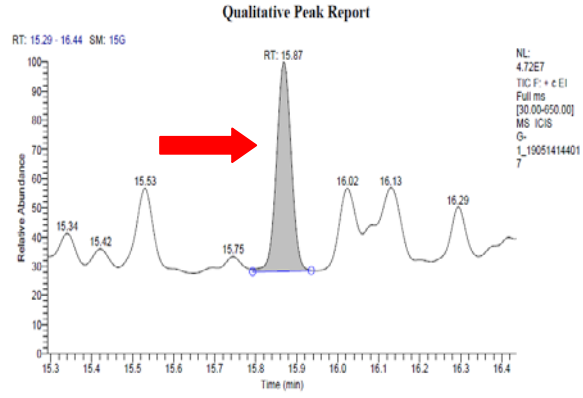

Figure 6. The GC-MS chromatogram of pentadecane compounds

The results in the figure above showed that pentadecane metabolites with the molecular formula $C_{15}H_{32}$ are a group of essential oils whose presence in plants functions as metabolites. Conversely, the pentadecane compounds released by plants have the potential to cause negative effects on the growth of surrounding plants. Furthermore, several essential oil components screened for allelopathic activity showed growth inhibition in *Lactuca sativa* L. and *Lactuca perenne* L. (Jones, 2012).

The results of the GC-MS chromatogram for the compound 9-Octadecenoic acid (Z, Z) are presented in Figure 7.

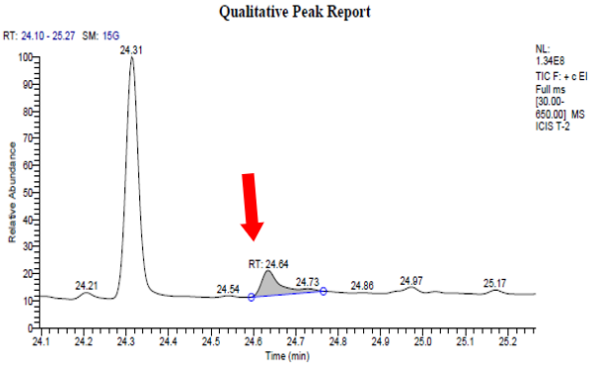

Figure7. The GC-MS chromatogram for the compound 9-Octadecenoic acid (Z, Z)

The results in Figure 7 above showed that the *S. officinarum* and *M. invisa* rhizosphere contained 9,12-octadecadienoic acid (Z, Z), methyl ester (3.86 %) compounds. Additionally, the *S. officinarum* root rhizosphere extraction contains the allelochemical compound 9,12-octadecadienoic acid (Z, Z) which has the molecular formula $C_{19}H_{34}O_2$ and is known to inhibit plant's growth. Karimi *et al*., (2011) stated that the ethyl acetate extract of walnut tree roots containing n-hexadecanoic acid, 9, 12-octadecadienoic acid (Z, Z), and 8-octadecenoic acid was able to suppress the germination and growth of cabbage (*Brassicaoleracea* L. var. capitata) seedlings (Chauhan, 2008).

4. Discussion

The results of the GC-MS analysis showed that the extracts of *S. officinarum* and *M. invisa* in the environment of different rhizosphere ecosystems contained different secondary metabolite compounds. This is presumbly due to the different conditions of the plant rhizosphere ecosystem making the compound contents within also different. Therefore, the research results showed that the diversity of secondary metabolite compounds was influenced by the environment in which the plants grew, specifically the soil nutrient content and the type of material to be identified (Asadi *et al.,* 2019; Sirikantaramas, 2008).

Furthermore, plants were shown to have growth organs that contained metabolite compounds. Conversely, the process of synthesis of plant secondary metabolite compounds is influenced by several factors including genetics, environmental stress, and physical factors (Aliyu *et al*., 2018; Andrew *et al*., 2014; Darmanti, 2018). Additionally, the difference in the content of these compounds is due to the different metabolite synthesis patterns between *S. officinarum* and *M. invisa*. The results of the identification of metabolite compounds in the four samples contained the same pattern of interaction of metabolites between these two plants for several compounds.

Consequently, the rhizosphere of *M. invisa* contained a dodecane allelopathic compound (6.83 %), which is a plant metabolite compound that has a molecular formula of $C_{12}H_{26}$ and a molecular weight of 170.33gmol^{-1} (Ram *et al*., 2019; Karimi *et al*., 2011). This dodecane compound is classified as essential oil which has the chemical name dihexil and is used in micro-extraction solvents for the formation of Triazine herbicide (Kokosa, *et al*., 2019). Similarly, this triazine is an active ingredient of herbicides used to control broadleaf and grassy weeds. Additionally, compound 2-Furan carboxaldehyde, 5-methyl with the molecular formula $C_6H_6O_2$, found in weed root extracts, is a class of furfural compounds that has allelopathic potentials in plants (Ram *et al.*, 2019). Moreso, pentadecane metabolite compounds, with the molecular formula $C_{15}H_{32}$, is a group of essential oils in plants functioning as metabolites with the potential to cause negative effects on the growth of plants surrounding them. Conversely, it was also shown that some components of essential oils which were screened for allelopathic activity showed growth retardation (Enyiukwu and Ononuju, 2016; Karimi *et al*., 2011; Lucas *et al*., 2021).

Furthermore, allelochemicals do not affect the activity of cells that synthesize and store them because plants have a mechanism of resistance to self-produced toxic compounds (Aliyu *et al*., 2018; Asyraf and Micheal, 2011; Chauhan, 2008). This resistance mechanism occurs through a biosynthetic process outside the cell such as in the secretory cell wall, storing it in vacuoles. Other than that, the allelochemical is transported from the cytoplasm to the vacuole by vesicles, where they experience enzymatic detoxification. Conversely, there is a mutation in the gene encoding the protein at the target sites of allelochemicals leading to a non–toxic accumulation of these chemicals in the vacuole (Erida *et al*., 2019; Marvillo *et al*., 2011; Sirikantaramas *et al*., 2008). Further, the allelochemical compounds released by roots into the soil can cause growth disorders in plants depending on the concentration of the allelochemicals. Additionally, these chemicals, when released into the environment, result in interactions between its biotic and abiotic factors such as physical-chemical processes, absorption by plants, and microbial breakdown (Albuquerque *et al*., 2011; Darmanti, 2018).

Consequently, chemicals that are secreted from plants that affect other plants are called allelochemicals (Riajeng, 2019; Suresh *et al*., 2017). Moreover, these chemicals are secondary metabolites produced by plants that can inhibit or stimulate the growth of plants in the vicinity. Therefore, most of these chemicals are classified as plant secondary metabolites which are produced from primary metabolites. A wide variety of these chemicals has been identified including phenolic acids, coumarins, terpenoids, flavonoids, alkaloids, glycosides, and gluconates (Karimi *et al*., 2017). Additionally, potential allelochemicals are found in almost all plant tissues, including the leaves, flowers, fruits, seeds, and root tissues. Also for decades, these chemicals were mostly used in agriculture, forestry, ornamental plants, and gardening. The results showed that they contain several naturally occurring chemical substances such as alcohol, organic acids, aliphatic and aromatic components.

Moreover, the effect of competition in plant communities can occur indirectly on individual plants, and this is usually due to allelochemical interactions. Therefore, based on the results of this research and discussion, it is concluded that the allelochemicals secreted

by donor plants will be released into the environment through root exudation by the process of diffusion, evaporation from leaves, leaching, and biomass decomposition (Euis and Ratag, 2017; Mohammad *et al*., 2021; Sirikantaramas *et al*., 2008). Similarly, the effect of these chemicals on seed germination, seedling growth gives it its allelopathic property. Therefore, allelopathy is a chemical component produced by plants to interact with other plants that grow around them (Erida *et al*., 2019; Lucas *et al*., 2021). It is used by weeds to compete with other plants that grow around it. The results of this research showed that weed extract can inhibit the germination of seeds of other plants growing in its vicinity, giving it the potential to be developed as a bioherbicide.

5. Conclusion

It can be concluded, based on the results of this research, that the *S. officinarum* rhizosphere contains an octadecanoic acid allelochemical compound, and methyl ester (2.06 %). Additionally, weed roots and *S. officinarum* each contained metabolite compounds which are usually used as herbicide activators.

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Characterization of Actinomycetes Strains Isolated from Cheliff Estuary in the North-West of Algeria

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Abstract

This study focused on the investigation of Actinomycetes strains isolated from sediment sample of Cheliff River estuary in Mostaganem (North-west of Algeria). Identification of the 5 strains of Actinomycetes isolated from sediments by morphological, biochemical methods and by16S rRNA gene sequence analysis revealed that four strains belonged to the genus *Streptomyces* and one - to the genus *Nocardia.* No strains of Actinomycetes were isolated from water samples. The optimum growth of all strains was recorded between 28°C and 30°C and at pH 7-9. All strains had halotolerant and mesophile behavior except SSG which grew very well at 45°C and could be considered as thermotolerant bacteria. Antibacterial activity was tested against five pathogenic bacteria as *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Salmonella typhi* ATCC 9289, *Bacillus subtilis* ATCC 6633 and *Pseudomonas aeruginosa* ATCC 2785. All isolated strains exhibited an inhibitory activity against at least one human pathogenic bacterium. *Streptomyces* sp. strain SSG showed antibacterial effect against four pathogenic tested bacteria followed by *Streptomyces sp.* SSB1, *Streptomyces sp.* SSM and *Nocardia sp.* NSR. This study revealed that Cheliff River estuary contains Actinomycete species (*Streptomyces* and *Nocardia*) with proved antibacterial activity and could be a new source for isolation of antibiotic producing bacteria against human pathogenic bacteria.

Keywords: Actinomycetes, Cheliff River, Estuary, Sediments, Phylogeny, Antimicrobial activity.

1. Introduction

Marine microorganisms such as *Actinobacteria* are important sources of biomolecules (Shepherd *et al.*, 2010; Blunt *et al.*, 2016; Giraou *et al.,* 2019). This important microbial group has been explored for biotechnology and provides excellent sources of medication especially against multidrug resistant pathogens (Abdelfattah, 2016; Newman, 2016; Ramachandran *et al.,* 2019). Previous studies were focused on new bioactive natural compounds from unexploited habitats (freshwater, mangrove, invertebrate, macroalgae) to find out potential diseases therapy (Ganesan *et al.*, 2016, Rotich *et al.*, 2017; Rangseekaew and Pathom-aree, 2019). Aquatic environment provides interesting prospects to find out new bioactive components (Gomez-Escribano *et al.*, 2016, Lee *et al.,* 2018). The *Actinobacteria* are widespread in various environments, and their presence in estuarine ecosystem might be promoted probably by the high nutrient content of this setting (Rosmine and Sramma, 2016). *Actinobacteria* are Gram-positive bacteria known to produce a panel of natural compounds exhibiting a wide range of biological activities (Prasad *et al.,* 2013; Abdelfattah, 2014; Sharma, 2016; Gamaleldin *et al*., 2020). A single actinomycete strain can produce from 10 to 20 different secondary metabolites molecules (Sosio *et al.*, 2000; Bentley *et al.*, 2002) such as antibiotic (Tetracyclines) (Hopwood, 2007); antifungal (Amphotericin), anticancer (Adriamycin) and immunosuppressant molecules (Tacrolymus).

Our work was oriented to the estuary of Cheliff River, which is one of the longest in Algeria. It takes its source from Algerian Sahara Atlas and empties into the Mediterranean Sea, across 733 km (Figure 1). We isolated five strains: for *Streptomyces* and one *Nocardia* strain, from estuarine sediment of Cheliff River, characterized using polyphasic taxonomy (using phenotypic and genotypic approaches) and investigate their potential to produce antibacterial molecules.

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2. Material and Methods

2.1. Sampling

Sediment samples from Cheliff estuary (36°02'18.8"N 0°08'04.8"E) were collected aseptically in duplicates. After *in situ* pH and temperature were measured, sediment samples were collected by sterile scraper at about 50 cm deep below the estuarine floor using sterile scrapper surface and aseptically transferred to sterile polyethylene bags. All samples were directly transferred in travel cool box for further microbiological analysis, performed immediately after arrival to the laboratory.

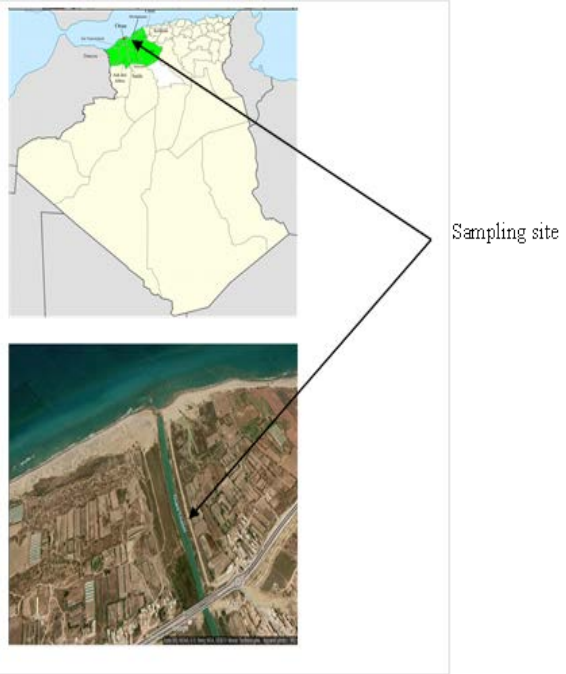

Figure 1: Cheliff River estuary geography location (Google Earth version 7.3, image © 2021 Maxar Technologies. Data SIO. NOAA. U.S.Navy. NGA. GEBCO)

2.2. Isolation and purification of actinomycetes

Prior to Actinomycetes isolation and in order to promote their growth, all the samples were oven-heated at 55°C and 1% of phenol was added for 6 min.

Actinomycetes isolation was performed using serial dilution method. One gram of sediment was suspended in 9 mL of Cheliff estuary water sterilized and diluted up to 10^{-4}. One milliliter of each dilution was spread on the surface of Petri dishes containing 20 mL of the solid culture media. Five different culture media (Starch Casein Agar, Glucose Asparagine Agar, Yeast Extract Glycerol Agar, Yeast Extract Glycerol Agar, malt extract- yeast extract agar (ISP-2: International *Streptomyces* Project-2 medium; composition: yeast extract: 4g, malt extract: 10, glucose: 4g, agar: 20g, pH 7.2) and malt extract-peptone yeast extract (GLM) were prepared using sterilized estuarian water amended with nalidix acid (10 µg/mL) and amphotericine B (25 µL/mL) sterilized on 0.22 µm filters (Ellaiah *et al.*, 1996; Cuesta *et al.*, 2010) and were tested to identify the most suitable one to study the actinomycetes. This different culture medium were amended with nalidix acid (10 µg/mL sterilized on 0.22 µm filters) and amphotericin B (25 µL/mL) to ovoid Gram-negative bacteria and fungi growth and to boost the actinomycetes development (Ellaiah *et al.*, 1996; Cuesta *et al.*, 2010). The inoculated Petri dishes have been incubated at 28°C for 2 weeks. The actinomycetes look-like colonies were then isolated and transferred on ISP-2 (Shirling and Gottlieb, 1966) agar plate medium for further characterizations. For short term preservation, monoclonal colonies on ISP-2 plates were stored at 4°C. For long term conservation, mycelia suspension was conserved with glycerol (50/50 v/v) and stored at -20°C (Sheperd *et al.*, 2010).

2.3. morphological, biochemical and physiological characterization

Actinomycetes strains were macroscopically characterized by the coloration of their areal mycelium and by the presence of diffusible pigments and morphological features. Gram staining was used both to distinguish the specific microscopic features and the Gram classification (Goodfellow *et al.*, 2004)

For biochemical characteristics, the API 20^{E} kit (bioMérieux Inc., Durham, NC) was used. Oxidase and catalase (Bactident ®Oxydase MERCK) activity were verified for *Streptomyces*-like bacteria. The effect of temperature, pH and salinity on the growth of the 5 strains was tested on Petri dishes solid ISP-2 media. Different incubation temperatures (4, 30, 37 and 45°C) and three pH values (5, 7 and 9) were tested using ISP-2 culture media. Salinity tolerance tests were carried out with ISP-2 supplemented respectively with 1, 3, 5, 7 and 10% of sodium chloride. Petri dishes were then streaked with actinomycetes monoclonal isolated strains and incubated at 30°C for 7 days. Daily visual observations were done for growth records.

2.4. Molecular characterization

Analysis of the 16S rDNA sequence and construction of phylogenetic tree using the neighbor joining method were used to confirm the actinomycetes identification. DNA extraction was done using the CTAB method associated to the boiling DNA from 3 days old strain cultures grown in 10 mL ISP-2 liquid medium (Cook and Meyers, 2003). The 16S rDNA amplification was performed using universal primers 9F (5'GAGTTTGATCMTGGCTCAG 3') and SQ6 (5'CGGTGTGTACAAGGCC3') (Weisburg *et al.*, 1991).

The final volume of the PCR reaction was 50 µL including Taq Buffer (Promega) 1/10, dNTPs (0.4 mM), $MgCl_2$ (4 mM) and 0.6 U Taq polymerase (Thermoscientific, UK), and 10 nM of forward and reverse primers. PCR was performed according to the following program: initial denaturation at 98°C for 3 min, followed by 30 cycles of reaction with denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C and final extension at 72°C for 10 min. The amplicons were sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems, USA) with the same primers as above to generate a nearly complete gene sequence (1167 to 1250). The 16S rRNA sequences were compared to database in NCBI -GenBank using Basic alignment Search tool (BLAST) (Altschul *et al.*, 1990). Multiple sequence alignment was compared with the sequences generated in this study using CLUSTAL W program (Larkin *et al.*, 2007). Phylogenetic evolutionary tree was conducted using the neighbor-joining methods in Molecular Evolutionary

genetic Analysis (MEGA version 6.0) software based on boostrap values of 100 replications (Tamura *et al.*, 2013).

The Genbank/EMBL/DDBJ accession number of the 16S rDNA sequences of the isolate SSB1 is MH398042, for the strain SSB2: MH398493, for the strain SSG: MH398494 for the isolate SSM: MH398495) and for the strain NSR: MH398496.

2.5. Antimicrobial activity tests

The antibacterial activity of the 5 isolated actinomycete strains was tested against five human pathogen bacterial strains: *Pseudomonas aeruginosa* ATCC 27857 (*P. aeruginosa*), *Escherichia coli* ATCC 25922 (*E. coli*), *Salmonella typhi* ATCC 9289 (*S. typhi*), *Staphylococcus aureus* ATCC 6538 (*S. aureus*) and *Bacillus subtilis* ATCC 6633(*B. subtilis*) kindly provided by the Pediatric Hospital of Oran (Algeria). One milliliter of the human pathogens ATCC standardized bacterial inoculum (10^6 UFC/mL) was incorporated to melted Mueller-Hinton agar in Petri dishes. Cylinders cut from cultures of our 5 strains grown on agar medium were inverted deposited on the surface of the Petri dishes containing the human pathogens; and were then incubated at 37°C for 24 hours. Inhibition zone diameter was measured.

2.6. antimicrobial metabolites production through the cultivation time

We used wells agar diffusion assay method on the same pathogenic ATCC bacterial strains previously used for antibacterial tests to investigate the inhibitory action of the 5 isolates on the growth of pathogenic strains. Crude supernatant (100 µL) obtained from 12 days old liquid cultures of our 5 isolated strains was dispensed aseptically into 8 mm diameter agar drilled wells. In addition, Streptomycin (0.2 µg/mL) used as a positive control was placed into the agar well; and sterile distilled water was also filled into a well and served as negative control. The assays were incubated at 37°C, and the bacterial inhibition zones around the well were visually controlled every 24 hours as clear zones around the wells. Their diameters were measured daily through the 12 days of the experiment.

3. Results and discussion

3.1. isolation and characterization

The pH of sediments sample varied from 7 to 8. The temperature at the collection site was 18°C.

Based on morphological characteristics (Figure 2), five different strains of actinomycetes (named SSB1, SSB2, SSG, SSM and NSR) were isolated from sediments. The dilution factor of 10^{-4} on ISP-2 medium gave the best monoclonal well separated colonies and allowed the isolation of the *Streptomyces* and *Nocardia* strains. In addition, all the strains were isolated in ISP-2. This result may be attributed to differences between the compositions of the different media. Higher amounts of complex nitrogen and carbon sources, such as yeast and malt extract may preferably promote the growth of estuarine *Actinobacteria* strains (Ozcan *et al.,* 2013).

The isolates grown on ISP-2 medium exhibited cream to white chalky colonies, with aerial and substrate multi-colored mycelia and have slow growth rate. The colonies were elevated, convex and powdery. Under optic microscopy, hook-like structures were found only with the isolates belonging to *Streptomyces* genus. The hook-like structures are characteristic of the *Streptomyces* genus and their evidence on our colonies reinforced the morphological characterizations of the 5 isolates. *Streptomyces* strains are also known to be able to produce a large variety of pigment responsible for the coloration of aerial and substrate mycelia which can be considered as a criterion for their classification using ISCC-NBS color chart (Kelly and Judd, 1955).

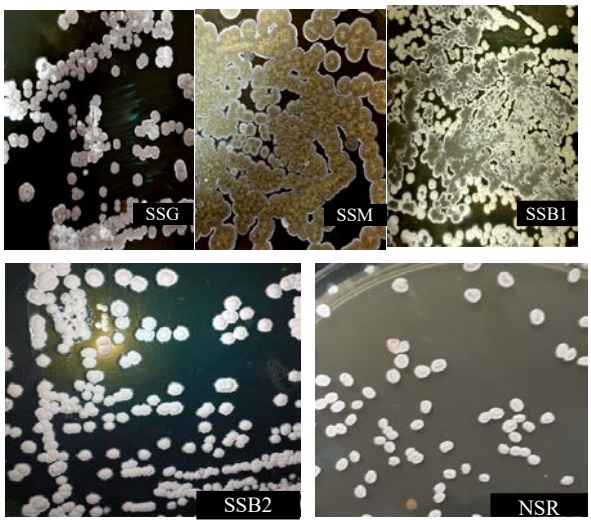

Figure2.Agar plates showing the color and morphology of the colonies of *Streptomyces* strains isolated from estuarian sediments. SSG: *Streptomyces* sp. G; SSM: *Streptomyces* sp. M; SSB1: *Streptomyces* sp. B1; SSB2: *Streptomyces* sp. B2; NSR: *Nocardia* sp. R.

3.2. biochemical and physiological characterization

API 20^E plate results (table 1) showed that all the strains were β-galactosidase producers except strain SSM. Strains SSB1, SSB2 and SSG possessed ADH and ODC enzymes and produce acetoin. SSB1, SSB2 and SSG strains were citrate positive. SSG was lysine decarboxylase positive. All strains were catalase, gelatin and indole positive but oxidase negative. Except for strain SSG, all other strains were gelatin and indole negative.

In addition, the availability of using various carbon substrates was tested, and the results varied among the isolates. All the strains used amygdalin as source of carbon. Strain SSM did not produce tryptophane desaminase and all of them were urease negative. Only strain SSG produced melanoid pigment. Comparing the five isolates, we considered that all strains were different from each other. However, the strains SSB1, SSB2 and SSG presented closely biochemical profile of carbon use (sorbitol, rhamnose, sucrose, melobiose, amygdalin and arabinose) and enzyme production (galactosidase, arginine hydrolase and ornithine decarboxylase). The use of different carbon sources by all strains indicated a wide pattern of carbon assimilation (Valan Arazu *et al.,* 2013). Most actinomycetes are known to use a wide range of organic compounds as carbon source for their growth.

Table 1. Characterization of the biochemical activity of the isolates using the API 20E test (BioMérieux Inc., Durham, NC)

Activity	Substrate	SSB1	SSB2	SSG	SSM	NSR
β-galactosidase	ONPG	+	+	+	-	+
Arginine dihydrolase	ADH	+	+	+	+	-
Ornithine decarboxylase	ODC	+	+	+	-	-
Acetoin production	VP	+	+	+	-	-
Citrate use	CIT	+	+	+	-	-
Lysine decarboxylase	LDC	-	-	+	-	-
H_2S production	H_2S	-	-	-	-	-
Urease	URE	-	-	-	-	-
Tryptophan desaminase	TDA	-	+	+	-	+
Indole	IND	-	-	+	-	-
Gelatinase	GEL	-	-	+	-	-
Glucose	GLU	-	+	-	-	-
Manitol	MAN	+	+	-	+	-
Inositol	INO	-	-	+	-	-
Sorbitol	SOR	+	+	+	-	-
Rhamnose	RHA	+	+	+	+	-
Sucrose	SAC	+	+	+	-	+
Meliobiose	MEL	+	+	+	-	-
Amygdalin	AMY	+	+	+	+	+
Arabinose	ARA	+	+	+	-	-
Melanoid pigment		-	-	+	-	-
Catalase		+	+	+	+	+
Oxydase		-	-	-	-	-

The growth temperatures tested (table 2) indicated that the strains SSB1 and NSR were able to grow from 7 to 37°C, SSB2, SSM from 28 to 37°C; while the strain SSG was able to grow from 28°C to 45°C. No growth, even moderate, was found at temperature below 28°C. All strains were mesophile except strain SSG which can be considered as thermotolerant bacteria. The thermotolerant actinomycetes play a significant role in the mineralization of nutrients and degradation of organic matter (Nayaka *et al,.* 2020).

Most of the strains were mesophiles, neutrophiles, growing in NaCl concentration ranged between 1 % and 10 %. However, some species showed a wide range of tolerance, like the eurythermal NSR strain which was psychrotolerant and mesophile. In terms of pH, the strains SSB2, SSG, SSM and NSR were acidotolerant and alkali-tolerant. Also, all the strains were halotolerant which can be explained by the fact that estuary is a transition zone between two aquatic ecosystems: fresh and marine water. Estuarine systems are affected by riverine and marine influences such waves, influx of saline water, flows of fresh water and sediment which create an unstable environment (Sobha *et al.,* 2014). Our strains developed an adaptive capacity to balance the osmotic pressure of the environment. This phenomenon was reported by many studies (Enache and Kamekura 2010; Caton *et al.,* 2009; Elshahed *et al.,* 2004). Moreover, physiological and biochemical characteristics are directly related to the enzymatic activity of the bacteria and to the regulation proteins (Li *et al.,* 2016). Morphology, metabolism and physiology of *Actinobacteria* differ from one species to another so they can be considered as strong tools for *Actinobacteria* differentiation and taxonomy (Pridham *et al.,* 1958; Wink *et al.,* 2016).

Table 2: Effects of temperature, pH and salinity on the growth of isolated strains (SSB1, SSB2, SSG, SSM and NSR)

		SSB1	SSB2	SSG	SSM	NSR
Temperature (°C)	7	++	-	-	-	++
	28	+++	+++	+++	+++	+++
	37	+++	+++	+++	+++	+++
	45	-	-	+++	+	+
pH	4.9	-	+	+	+	+
	7	+++	+++	+++	+++	+++
	9	++	+++	+++	+++	+++
NaCl (%)	1	++++	++++	++++	++++	++++
	3	+++	++++	++++	+++	+++
	5	+	++++	++++	++++	++
	7	+	+++	++++	+++	+
	10	+	+++	++++	++	+

- : No growth; +: poor growth; ++: Moderate growth; +++**:** Good growth; ++++**:** abundant growth.

3.3. molecular characterization

Following the sequencing of the entire 16S rRNA gene of the five isolated strains, the comparison with sequence available in the NCBI database the BLAST analysis showed that all the strains were related to the *Actinobacteria* phyla. The isolates SSB1, SSB2, SSG and SSM were affiliated within the *Streptomyces* genus. Indeed, the strain SSB1 (GenBank accession number MH398042) exhibited 92.50% of similarity with *Streptomyces pratensis* type strain and *Streptomyces cyaneofuscatus* type strain. The isolate SSB2 (GenBank accession number MH398493) showed 96.55% of similarity with *Streptomyces pactum* type strain, while the strain SSG presented (GenBank accession number MH398494) 96.54% of similarity with *Streptomyces olivaceus* type strain. The strain SSM (GenBank accession number MH398495) showed 95.67% of similarity with *Streptomyces intermidius* type strain. The fifth isolated strain NSR (GenBank accession number MH398496) was assigned to *Nocardia* genus, by presenting 95.29% of similarity with *Nocardia thailandica* type strain. It was placed with three strains: *Nocardia thailandica* type strain, *Nocardia neocaledonensis* type strain and *Nocardia asteroides* type strain. Comparing the data, the strain NSR was biochemically, physiologically and phylogenetically distinct from *Nocardia thailandica* type strain. Strains SSB2 and SSG were close to each other and share 97, 09% of similarity. The two other sequences related to the *Streptomyces* genus were widespread in the phylogenetic

tree and were grouped in 2 different clusters. The first group encompassed the SSB1 sequence which was closely related to *Streptomyces pratensis* type strain, *Streptomyces cyanofuscatus* type strain and *Streptomyces lavendulae* type strain, while the second group with the SSM sequence was located between *Streptomyces distaticus* (Krainsky, 1914) and *Streptomyces intermidus* (Waksman et Lechevalier, 1953) and *Streptomyces rutgersensis*16s rRNA gene sequences. The phylogenetic tree showed that the *Streptomycete* isolates (SSB1, SSB2, SSG and SSM) were distributed in different clusters. Moreover, they exhibited a significant difference with phenotypical, biochemical and physiological characteristics.

To distinguish between the closely related species of the genus *Streptomyces* and *Nocardia* cited above in tree, the polyphasic approach (Vandamme *et al.,* 1996; Goodfellow *et al.,* 2004) such as morphological, physiological, biochemical including genetic characterization were used to identify and classify the isolated species.

Then, comparing the physiology, metabolic activities and 16S rRNA gene sequences of the isolates SSB1, SSB2, SSG, SSM, with the nearest *Streptomyces* strains and NSR with the closest *Nocardia* strains. The results show that each of our isolated strains have less than 97% of 16S rRNA gene similarity from their closest relatives in addition to several differentiating features (profile of carbon use, production of ADH, ODC, LDC and tyrosinase enzymes and culture characteristics). We deduct that the isolated strains SSB1, SSB2, SSG, SSM and NSR might be new species (Janda and Abbott, 2007).

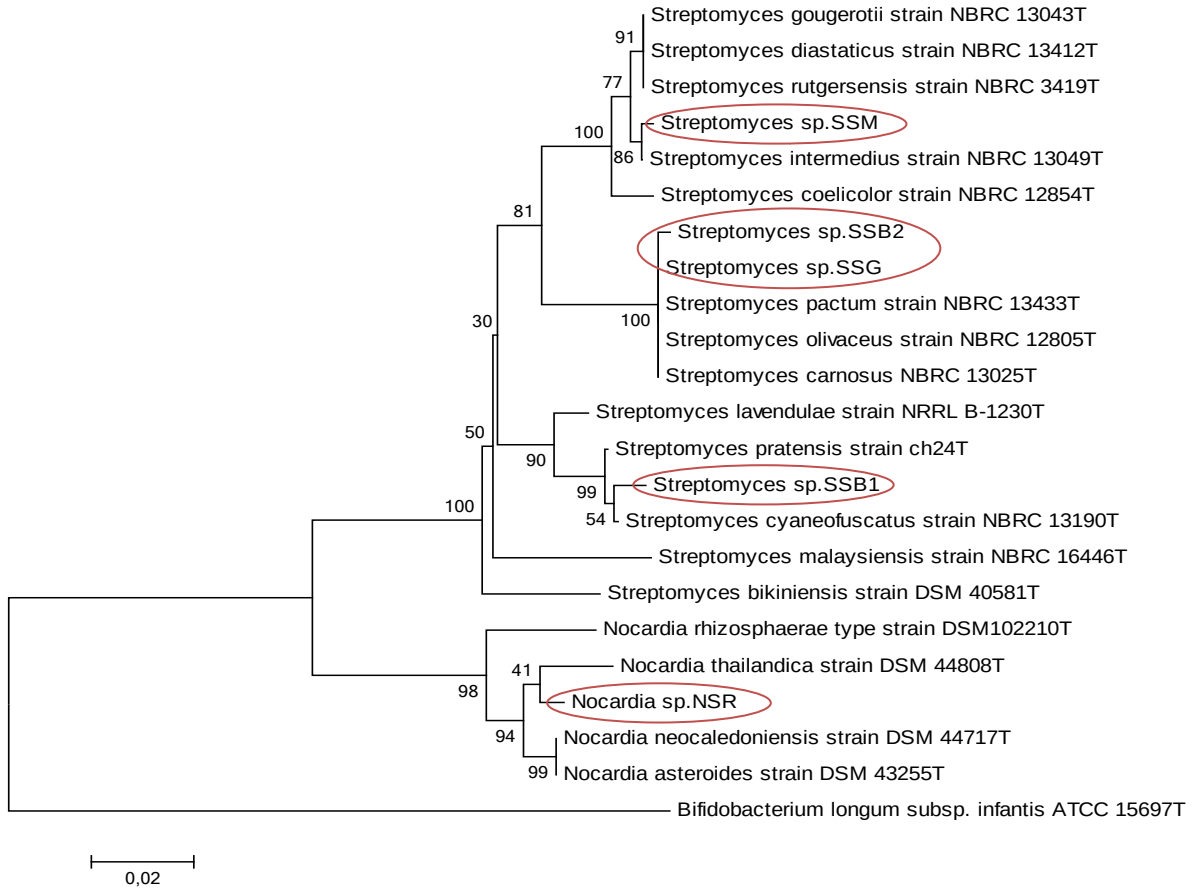

Figure 3. Phylogenetic tree based on 16S rRNA sequences of actinomycetes isolates (*Streptomyces* sp. **SSB1**, *Streptomyces* sp. **SSB2,** *Streptomyces* sp **SSG,** *Streptomyces* sp **SSM** and *Nocardia* sp. **NSR**) using the Neighbor-Joining method (Saitou and Nei, 1987). The number on branch nodes were boostrap values (from 100 replicates) (Felsenstein, 1985). Evolutionary analyses were performed in **MEGA 6.1.** (Tamura *et al., 2013*). The genus *Bifidobacterium* type strain was used as an out-group.

3.4. antimicrobial Activity

The results of the screening for the antibacterial activity were represented in table 3. Strains SSB1, SSG, SSM and NSR exhibited different spectrum of antibacterial activity (table 3). *Escherichia coli* ATCC 25922 was inhibited by four isolates (SSB1, SSG, SSM and NSR), while all strains did not show any antibiosis activity against *P. Aeruginosa* ATCC 27857. Among the 5 isolates, strain SSG exhibited the largest antibacterial activity as it inhibited the growth of all tested pathogenic strain at the exception of *Staphylococcus aureus* ATCC 6538. The antibiotic resistance shown by *S. aureus* ATCC 6538, for which the growth was inhibited by none of the tested strains, was probably due to mutations by a modification in ribosomal RNA methylase (Heelan *et al.*, 2004; Martinez *et al.,* 2018), by acquisition of resistance genes (plasmid-

mediated resistance) (Foster, 2017), by the presence of Staphylococcus biofilm's (Craft *et al.,* 2019; Saxena *et al*., 2019).

Table 3. Maximal diameter values in millimeters of the inhibition zones displayed by the 5 isolated Actinomycetes against pathogenic ATCC bacterial strains

	ISOLATED BACTERIAL STRAINS					CONTROLS	
	SSB1	SSB2	SSG	SSM	NSR	Positive	Negative
TESTED BACTERIA							
Escherichia coli ATCC 25922	8	-	8	7	10	+	-
Staphylococcus aureus ATCC 6538	-	-	-	-	-	+	-
Salmonella typhimurium ATCC 9289	4	-	11	-	-	+	-
Bacillus subtilis ATCC 6633	3	-	12	-	-	+	-
Pseudomonas aeruginosa ATCC 14028	-	-	8	-	-	+	-

The largest inhibition zones were obtained with the strain SSG against 4 bacterial pathogenic species; *B. subtilis* ATCC 6633, *S. typhi* ATCC 9289, *E. coli* ATCC 25922, *P. aerugenosa* ATCC 14028 (8-12mm, figure 4). Antibacterial activity was early observed with *E. coli* ATCC25922. In fact, after 24 hours of incubation, the maximum of bacterial growth inhibition reached and then decreased gradually, to finally disappear at day 9. *Streptomyces* strains are known to produce bacteriocin as a protein produced by ribosomes with large bactericidal spectrum activity against both Gram negative and positive bacteria (Jung *et al.*, 1992; Lee *et al.*, 2014; Hernandez-Saldana *et al*., 2020). Antibacterial activity of the strain SSG against *B. subtilis* ATCC 6633 occurs after 2 incubation days and after 4 days against *S. typhi* ATCC 9289. This bioactivity was optimal at day 7 and decreases gradually after. Likewise *P. aerugenosa* ATCC 14028 was susceptible to strain SSG bioactive compounds after 6 incubation days with maximum inhibition effect at day 10 and then decreases. Owing that the antibacterial activity of the strain SSG beginning at day 1, against only one of the bacterial strains tested (*Escherichia coli* ATCC 25922) and then through the incubation time, affecting the other bacterial strains, we suspected that a mix of bioactive metabolites was produced by the isolate SSG at different bacterial growth steps. *Streptomyces* are known to produce antimicrobial secondary metabolites, as single or mix (Macagnan *et al.*, 2006; Procopio *et al.*, 2012; Song *et al.,* 2013; Song *et al.,* 2015). Previous studies showed that different strains of *Streptomyces albus* possess different gene cluster encoding the production of specific secondary metabolites (Seipke, 2015).

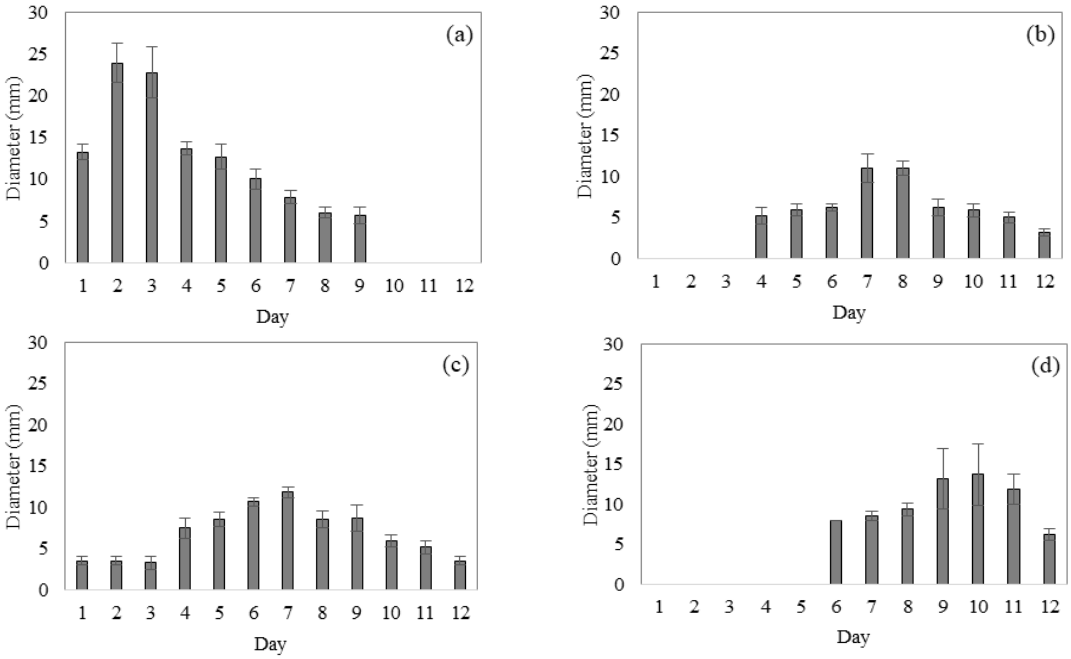

Figure 4: Antibacterial production of strain SSG through the cultivation time: **(a) against** *E. coli*.**(b) against** *S. typhi*. **(c) against** *B. subtilis*. **(d) against** *P. Aeruginosa*

4. Conclusion

This work showed that the studied estuarian actinomycete strains could be new species of the genus *Streptomyces* and *Nocardia.* On the basis of phenotypic characteristics, growth parameters (temperature, pH, salinity), metabolic and antimicrobial activity in addition to the 16s rRNA gene sequences analysis of the 5 isolated strains (SSB1, SSB2, SSG, SSM and NSR) we conclude

that these ones can be new strains belonging to *Actinobacteria* phyla. The strains SSB1, SSB2, SSG and SSM were affiliated within the *Streptomyces* genus and strain NSR within *Nocardia* genus. Owing the 16S rRNA gene sequence similarity with their closest neighbor sequence, the strains SSB1, SSB2, SSG, SSM and NSR could be considered as 5 novel species. However, more analysis, like DNA-DNA hybridization is needed to prove that. In addition, our investigation showed that the estuarine sediment harbored actinomycetes that could produce secondary metabolites with antibacterial activity.

The optimization of the culture conditions (temperature, shaking, pH and medium composition) was conducted (data not shown), and the characterization of the antimicrobial molecules is in progress. Further purification, characterization and structural elucidation of biomolecules are needed to explore the antimicrobial activity of these molecules.

5. Compliance with ethical standards

6. Conflict of interest

The authors declare that they have no conflict of interest.

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Biological Activities and Metabolite Profiling of *Polycarpa aurata* (Tunicate, Ascidian) from Barrang Caddi, Spermonde Archipelago, Indonesia

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Abstract

The U.S. Food and Drug Administration (FDA) has legalized several bioactive compounds from marine organisms, and two of them were isolated from tunicate (ascidians). However, the bioactive compounds from marine tunicate are less reported than other marine organisms. This study was conducted to screen biological activities and secondary metabolite of Indonesia's marine tunicate *Polycarpa aurata* from Barrang Caddi, South Sulawesi. Sample was extracted using methanol for 24 h by maceration. The bioactive compounds were characterized using phytochemical tests and HPLC-DAD. Antibacterial activity was performed against multidrug-resistant (MDR) Methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus*, *Salmonella typhi*, together with a non-MDR *Escherichia coli*, while antifungal against *C. albicans* and *T. rubrum*. Antioxidant activity was analyzed using DPPH method; and cytotoxicity against P388 murine leukemia cells using XTT method. The crude extract inhibited all pathogenic bacteria in its lowest concentration (0.5 mg) but did not show antifungal effect, toxicity at 0.08 mg/mL; antioxidant 534.60 ppm. The result of phytochemical test gave positive result for alkaloid and steroid/triterpenoid. In addition, HPLC chromatograms indicated 6 major peaks.

Keywords: antibacterial, cytotoxic, Leukemia, MDR bacteria

1. Introduction

In exploring novel medicine to treat various infections and diseases, marine natural product (MNP) has seized the world's attention through its incredible bioactive compounds (Blunt et al., 2018; Pereira, 2019). Their secondary metabolites exhibit potential biological activities such as antibacterial, anticancer, antitumor, antiviral, immunostimulant, etc (Blunt et al., 2018; Carroll et al., 2019; Hanif et al., 2019). Furthermore, Carroll et al., (2020) stated that bacteria, fungi, sponge, cnidarian, and algae were highlighted as the most productive source of new MNPs. For instance, 8 MNPs have been approved by European Medicines Agency (EMEA), Japanese Ministry of Health and Australia's Therapeutic Goods Administration, and U.S. Food and Drug Administration (FDA) (Pereira, 2019). In addition, several other MNPs such as Plinabulin, Plocabulin, and Salinosporamide A are still under clinical trial before legalized and approved by authorized institutions (Jiménez, 2018; Pereira, 2019).

Prior reports stated discovering new compounds from marine tunicates (ascidian) is less reported than sponge and cnidarian (Blunt et al., 2018; Carroll et al., 2019; Carroll et al., 2020). In 2018, there were only 27 new MNPs from tunicate (Carroll et al., 2019), while in 2019, only 12 new MNPs were recorded (Carroll et al., 2020). Despite the low number of new MNP from tunicate, the metabolites from this sessile animal exhibited outstanding biological activities (Arumugam et al., 2017; Leisch et al., 2019). Moreover, two tunicate-derived MNPs have been approved by internationally authorized institutions for drugs. Besides, other tunicate-derived compounds such as Didemnin B from *Tridemnum solidum* are examined in Phase II and Phase III for clinical trial as anticancer (Arumugam et al., 2017; Jiménez, 2018; Leisch et al., 2019). Most of the studies on biological activity from tunicate-derived MNPs are focused on anticancer; however, other properties are pretty neglected (Palanisamy et al., 2017). Hence, screening of biological properties from marine tunicate is essential.

Although Indonesia harbors various tunic species, a survey by Hanif et al. (2019) indicated a lack of MNP data from this animal. Palanisamy et al. (2017) stated that among all reported MNPs from marine tunicate, only 12% of them were isolated from family Stylidae. *Polycarpa aurata* is one of marine tunicate from family Stylidae that is scattered in the center and eastern part of Indonesia nevertheless, only a few studies reported its biological

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activity (Litaay, 2018; Litaay et al., 2018; Ayuningrum et al., 2019; Sumilat et al., 2019). Therefore, the study of biological properties of Indonesia's *P. aurata* is interesting to be conducted. This current study aimed to obtain a crude extract of *P. aurata* collected in Barrang Caddi, analyse the bioactive compounds, and study the biological properties such as antibacterial, antifungal, anticancer, and antioxidant.

2. Materials and Methods

2.1. Sampling

Sampling was conducted in Barrang Caddi waters, Spermonde Archipelago, South Sulawesi, Indonesia (Figure 1). *P. aurata* was collected by SCUBA diving. Samples were put into ziplock plastics, then transferred into a cold box to retain the quality then sent to Laboratory of Tropical Marine Biotechnology (TMB), Universitas Diponegoro, Semarang. The sample was identified by comparing the morphology to a previous report by Ayuningrum et al. (2019).

2.2. Metabolite Extraction

Samples were cleaned using flowing marine and freshwater. The internal organs were discarded while the other part (flesh) was resized and homogenized using a blender machine. The extraction was conducted using methanol (2:1 %v/w) then agitated using an orbital shaker for 24 h (Sibero et al., 2019[1]). The metabolites were concentrated using a rotary evaporator. Afterward, ethyl acetate was added into the unevaporated part then agitated for 1h and separated using separatory funnel. The ethyl acetate layer was collected, then evaporated to get the crude extract then kept at -20 before use for further analysis (Sibero et al., 2020[1]).

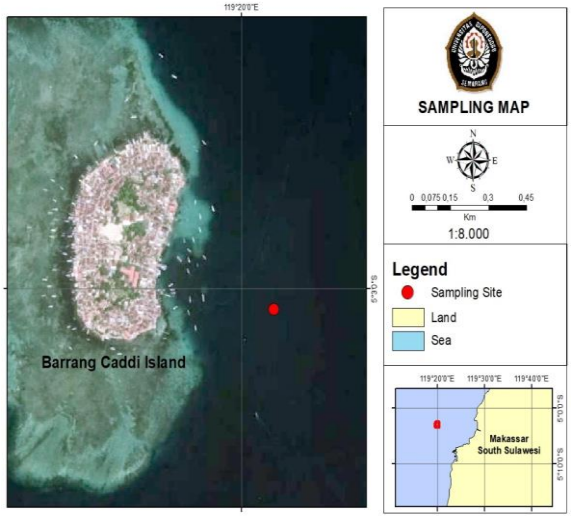

Figure 1. Sampling site in Barrang Caddi, Spermonde Archipelago, South Sulawesi, Indonesia

2.3. Bioassay

2.3.1. Antimicrobial activity

Antimicrobial activity consisted of an antibacterial assay against multidrug-resistant (MDR) *B. cereus*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi* and non-MDR *E. coli* obtained from Dr. Kariadi Hospital, Semarang, Indonesia; while antifungal assay against *C. albicans* and *T. rubrum* were from Toyama Prefectural University, Japan. The assay was conducted using the Kirby-Bauer disc diffusion method with a modification that has been published in our previous report (Sibero et al., 2019[1]; Sibero et al., 2020[1]). The crude extract was diluted in dimethyl sulfoxide (DMSO) with 4 concentrations (0.5; 1.0; 1.5 and 2.0 mg/mL) then 15 µL of each concentration was injected into paper disc (Advantec, Ø 6mm). Amoxicillin + clavulanic acid 30 µg/disc (Oxoid) was carried out as positive control for antibacterial and Cyclohexamide (Wako, Japan) 30 µg/mL was prepared as positive control for antifungal. The presence of a clear zone around the paper disc indicated the presence of antimicrobial.

2.3.2. Antioxidant activity

Antioxidant activity was determined using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical method according to Sejati et al. (2020). A total of 3.94 mg DPPH crystal was dissolved into 10 mL absolute methanol (Merck) to prepare a DPPH stock solution with a concentration 0.1 mM. Extract was dissolved in methanol to reach the concentration of 200, 400, 600, 800 and 1000 ppm. In total 160 µL of each concentration was transferred into a 96-wells plate, then 40 µL of DPPH stock was added to reach 200 µL/well. Then it was kept for 30 mins in an incubator (37 ºC) without any light exposure. Afterward, the absorbance was measured at 517 nm using a microplate reader with three replications. The IC_{50} values for antioxidant activity were calculated by linear regression method.

$$DPPH\ Scavenging\ effect = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

2.3.3. Cytotoxicity

The cytotoxic potential of *P. aurata* methanol extract was tested against P388 murine leukemia cells. This step was done according to our previous works with XTT cell proliferation assay (Sibero et al., 2020[1]; Sibero et al., 2020[2]). The crude extract was diluted in DMSO to reach concentration of 0.0002; 0.002; 0.02; and 2 mg/mL. Doxorubicin was used as positive control while DMSO as negative control. The cell viability was counted using a microplate reader at 540 nm. The results of experiments were plotted on single-logarithmic charts to deduce IC_{50} values.

2.4. Metabolite Profiling

2.4.1. Phytochemical test

The presence of bioactive compounds in the crude extract such as alkaloid, flavonoid, glycoside, saponin, steroid and triterpenoid were detected by phytochemical test, following our previous study (Sibero et al., 2019[1]; Sibero et al., 2020[1]; Sibero et al., 2020[2]).

2.4.2. High Performance-Liquid Chromatography with Diode Array Detector (HPLC-DAD)

Metabolite profile of *P. aurata* crude extract was carried out using HPLC-DAD (Sibero et al., 2020[1]; Sibero et al., 2020[3]). The crude extract was prepared in DMSO to reach concentration of 1 mg/mL, filtered using cosmonice filter pore size 0.45 µm (Nacalai Tesque, Inc) then injected into HPLC-DAD (Agilent 1100 Series). Acetonitrile (CH_3CN) and 0.1% formic acid buffer were applied as the

eluent while COSMOSIL 3C18-AR-II (4.6ID × 100 mm) from Nacalai Tesque was set as the column. The sample was analyzed with the following condition: 0-40 % of acetonitrile for 0- 25 min, 40-85 % for 25-28 min, 85 % for 28-30 min, and 85-90 % for 30-35 min with flow rate was 1.2 mL/min and pressure 160 bar. Metabolite in the crude extract was compared to the database in the computer.

3. Result

3.1. Sample identification

Barrang Caddis is one of the small islands in the Spermonde Archipelago, South Sulawesi Indonesia with abundance of marine resources. In this location, samples were collected at 5-10 m depth. The samples had an urn shape and two siphons at the top with yellow color in the inner part. Blue and white colors dominated the body while blue line color separated the compartment in the tunic (Figure 2). Samples were found attached on coral as solitary tunicate and co-presence with other organisms.

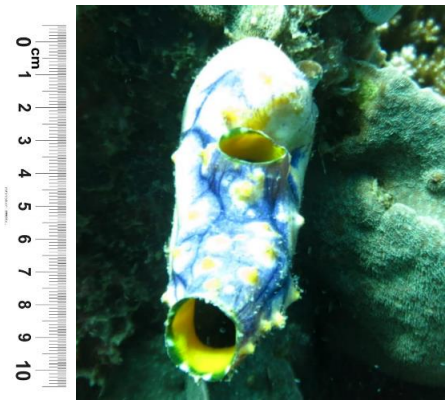

Figure 2. Morphology of *Polycarpa aurata* from Barrang Caddi, Spermonde Archipelago

3.2. Antibacterial activity

The samples were extracted using methanol to obtain the secondary metabolites then tested their biological activities. The antimicrobial activity of *P. aurata* is presented in Table 1, while Figure 3 shows the inhibition zone of each extract concentration.

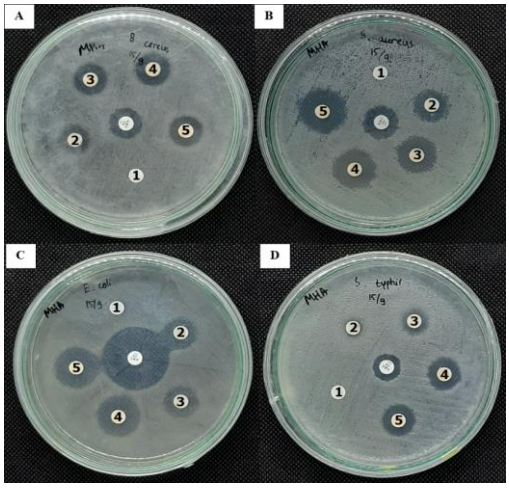

Figure 3. Result of antibacterial activity against pathogenic bacteria

(A. *Bacillus cereus*, B. MRSA, C. *Escherichia coli*, D. *Salmonella typhi*)

((1) DMSO, (2) 0.5 mg/mL, (3) 1.0 mg/mL, (4) 1.5 mg/mL, (5) 2.0 mg/mL)

Table 1. Antimicrobial activity of *P. aurata* crude extract against pathogens after 24 h incubation

Pathogen	Concentration (mg/mL)	Diameter of Inhibition Zone (mm)
Bacillus cereus	DMSO	0.00 ± 0.00[a]
	0.5	3.90 ± 1.50[b]
	1.0	5.90 ± 1.20[c]
	1.5	6.15 ± 0.21[c]
	2.0	6.65 ± 0.49[d]
	Amoxicillin + clavulanic acid 30 µg/disc	6.20 ± 0.70[c]
MRSA	DMSO	0.00 ± 0.00[a]
	0.5	7.05 ± 0.07[b]
	1.0	9.75 ± 0.35[c]
	1.5	9.95 ± 0.91[c]
	2.0	11.5 ± 0.75[d]
	Amoxicillin + clavulanic acid 30 µg/disc	7.80 ± 1.13[c]
Escherichia coli	DMSO	0.00 ± 0.00[a]
	0.5	6.30 ± 0.98[b]
	1.0	7.55 ± 1.48[b]
	1.5	11.65 ± 0.49[c]
	2.0	12.60 ± 1.41[c]
	Amoxicillin + clavulanic acid 30 µg/disc	21.65 ± 0.49[d]
Salmonella typhi	DMSO	0.00 ± 0.00[a]
	0.5	2.25 ± 0.77[b]
	1.0	5.75 ± 0.21[c]
	1.5	7.60 ± 1.41[d]
	2.0	7.10 ± 0.84[d]
	Amoxicillin + clavulanic acid 30 µg/disc	5.80 ± 0.28[c]
Candida albicans	DMSO	0.00 ± 0.00
	0.5	0.00 ± 0.00
	1.0	0.00 ± 0.00
	1.5	0.00 ± 0.00
	2.0	0.00 ± 0.00
	Nystatin 30 µg/mL	18.00 ± 0.00
Trichophyton rubrum	DMSO	0.00 ± 0.00
	0.5	0.00 ± 0.00
	1.0	0.00 ± 0.00
	1.5	0.00 ± 0.00
	2.0	0.00 ± 0.00
	Nystatin 30 µg/mL	18.00 ± 0.00

((Data were average ± standard deviation. Different notations indicate significant difference at $P < 0.05$)

The data (Table 1) shows that the crude extract of *P. aurata* inhibited all pathogenic bacteria; however, it did not show antifungal activity against *C. albicans* and *T. rubrum*. It was highlighted that the crude extract gave the best antibacterial activity against *E. coli*, followed by MRSA, then *S. typhi* while the narrowest antibacterial was shown against *B. cereus*. It was also noted that the enhancement of crude extract concentration was in line with increasing the inhibition zone diameter.

3.3. Antioxidant activity

Another important biological activity is antioxidants. The result of antioxidant activity of the crude extract is shown in Table 2 and Figure 4.

Table 2. Antioxidant activity of *P. aurata* crude extract

Concentration (ppm)	Inhibition (%)	IC_{50} (ppm)
200	21.63 ± 2,85	
400	44,23 ± 2.05	
600	58.42 ± 1.32	534.60 ppm
800	67.95 ± 1.19	
1000	80.93 ± 1.32	

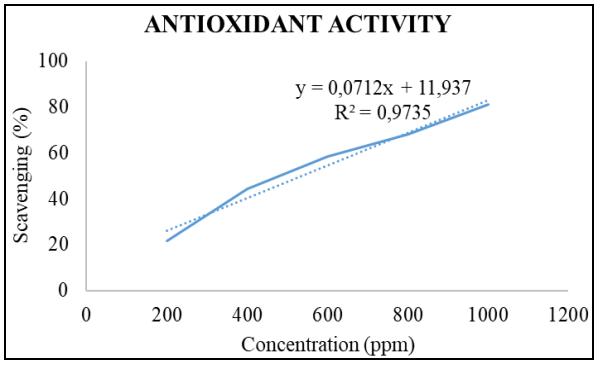

Figure 4. IC_{50} value of *P. aurata* crude extract using DPPH method

The ability of *P. aurata* crude extract on inhibiting DPPH was applied to screen the antioxidant activity. The result in Table 2 and Figure 4 shows that this extract had IC_{50} value of 534.60 ppm. It means that the crude extract scavenged 50% of DPPH activity at that particular concentration.

3.4. Cytotoxicity

The ability of *P. aurata* crude extract against P388 murine leukemia cancer cells is presented in Figure 5. It was noted that the crude extract exhibited anticancer potential with IC_{50} value of 0.08 mg/mL or equal to 80 µg/mL.

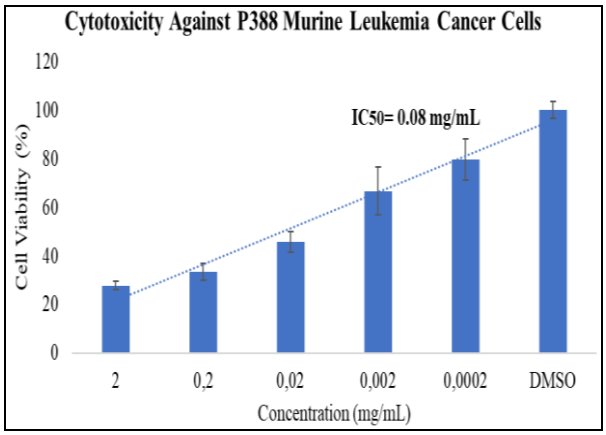

Figure 5. Cytotoxicity of *P. aurata* crude extract against P388 murine leukemia cancer cell

3.5. Metabolite profile

The presence of antibacterial, antioxidant, and cytotoxic properties leads to an understanding that *P. aurata* from Barrang Caddi is a potential source of bioactive compounds. Hence, profiling the secondary metabolites was conducted in this study. A fundamental analysis to detect the presence of bioactive compounds was performed using the phytochemical test. The result of this test is presented by Table 3.

Table 3. Phytochemical content of *P. aurata* crude extract

Target	Indicator	Result
Alkaloid	Formation of yellow to orange feculence after addition of Dragendorff reagent	Detected
Flavonoid	Formation of yellow to orange color in amyl alcohol layer	Not Detected
Glycoside	Formation of a brown ring between the layer	Not Detected
Saponin	Formation of a stable foam after 30 min and addition of 2N HCl	Not Detected
Steroid/Triter penoid	Formation of green color at the upper layer and maroon to brown color at the lower layer	Detected

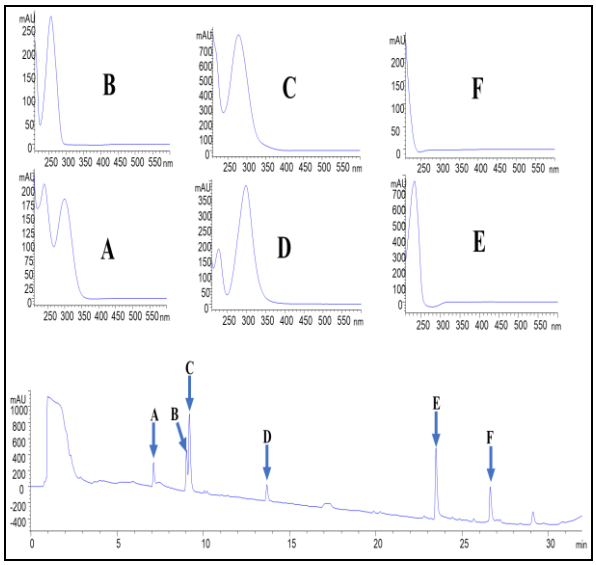

Figure 6. Metabolite profile of *P. aurata* crude extract using HPLC

According to the phytochemical test, the crude extract of *P. aurata* contained alkaloid and steroid/triterpenoid. Further, the HPLC chromatogram of the crude extract is presented by Figure 6. The HPLC analysis discovered 6 major peaks from *P. aurata* at retention time of 7.07 min (peak A), 8.97 min (peak B), 9.21 min (peak C), 13.71 min (peak D), 23.48 min (peak E) and 26.63 min (peak F).

4. Discussion

Morphology and molecular studies by Ayuningrum et al. (2019) successfully explained that the diverse color variations in *P. aurata* are probably induced by multiple allele inheritance with codominance. The existence of marine tunicate *P. aurata* in Barrang Caddi has been reported before, moreover, this species was noted as a cosmopolitan tunicate in Spermonde archipelago, South Sulawesi (Litaay, 2018). In addition, several tunic color variations of *P. aurata* are blue color with blue lines, orange color, yellow color with a blue line and white spots and blue lines (Ayuningrum et al., 2019). This species was also found in Indonesia, such as Bali, North Sulawesi, South Sulawesi, and Maluku (Wainwright et al., 2013; Litaay, 2018; Ayuningrum et al., 2019; Casertano et al., 2019; Sumilat et al., 2019). Even though this species has been studied widely, there are only a few reports about its biological activity, especially as antimicrobial and antioxidant agents.

The methanol crude extract was noted to possess alkaloid and steroid/triterpenoid (Table 3). Alkaloid derivatives compounds were commonly isolated from *P. aurata* such as N,N-Didesmethylgrossularine-1 (DDMG-1); polycarpaurines A-C; polyaurines A-B; and polycarpathiamines A-B (Wang et al., 2007; Pham et al., 2013; Casertano et al., 2019; Sumilat et al., 2019). Palanisamy et al. (2017) noted that most of the reported compounds from tunicates were alkaloid, consisting of indole 48%, pyrocridine 18%, β-carboline 8%, and saturosporine 5%. In addition, steroid only possesses 2% of the total tunicate-derived MNPs. Other chemical classes from marine tunicate were peptide 4%, alkene 3%, alkyl sulfate 3%, polyketide 2%, polysaccharide 2%, esters 1% and spiroketal 1%. In addition, HPLC chromatogram (Figure 6) endorsed the presence of secondary metabolites in *P. aurata*. The HPLC chromatogram and U.V. spectrum could be used to isolate the lead compounds from the sample. Previous studies successfully isolated new compounds from marine organisms through HPLC-UV guided isolation method (Sibero et al., 2019; Karim et al., 2020; Zhang et al., 2020). Furthermore, bioguided fractionation could also be combined with HPLC to obtain active fractionations based on the retention time (Sibero et al., 2020). Therefore, a further study is suggested to isolate the lead compounds. The presence of bioactive compounds in *P. aurata* crude extract is expected to influence its biological activities.

It was highlighted that the crude extract only inhibited the bacterial growth; however, it did not show any antifungal effect to *C. albicans* and *T. rubrum.* The ability of the crude extract on inhibiting gram positive pathogens (*B. cereus*, MRSA) and gram negative pathogens (*E. coli* and *S. typhi*) indicated the broad-spectrum antibacterial property (Kaur et al., 2011). Our data shows that the crude extract could inhibit MDR pathogens such as *B. cereus*, MRSA, and *S. typhi*. This result was expected to strengthen *P. aurata* as a prospective source of new antibiotics to overcome multidrug-resistance (MDR) bacterial infection. Although there are several studies about *P. aurata* crude extract's antimicrobial property nonetheless, there is no study about the lead compound of antibacterial activity within the past 5 years (Casertano et al. 2020). Palanisamy et al. (2017) stated that among all tunicate-derived MNPs, only 12% had antibacterial activity, and 3% had antifungal effect. Antifungal compounds combat the pathogens by inhibiting and or disrupt ergosterol, glucan, chitin, nucleic acids, and protein synthesis (Kathiravan et al., 2012). Some compounds reported to have antifungal activity are arylamidine, azoles, echinocandin, enfumafungin pyrrole, and quinazoline derivatives (Kathiravan et al., 2012; Castelli et al., 2016). These compound derivatives are barely isolated from *Polycarpa* spp. In addition, Casertano et al. (2020) stated that most antimicrobial compounds from ascidian are sulfur-containing compounds, meroterpenes, alkaloids, peptides, furanones derivatives. Therefore, only a few antifungal compounds were discovered from ascidian (Palanisamy et al., 2017).

The screening of antioxidant activity gave IC_{50} value of 534.60 ppm. A substance will be considered as a powerful antioxidant agent if it has IC_{50} values < 50 ppm, strong activity if the IC_{50} value is 50-100 ppm, moderate activity if the IC_{50} value is 100-150 ppm, and weak if the IC_{50} value is > 150 ppm (Haerani et al., 2019). Hence, our crude extract was not suggested as a candidate for a new antioxidant agent. Study of antioxidant property of marine tunicate is less popular than other biological activity. The latest update stated that only 2% of all reported compounds had antioxidant properties (Palanisamy et al., 2017). The presence of hydroxyl (HO) functional group in a compound is positively correlated to antioxidant activity; therefore, most phenolic-derivate compounds such as flavonoid have strong antioxidant activity (Chandra et al., 2020). Flavonoid was not detected in the crude extract; hence, it generally explains the infectivity of *P. aurata* as an antioxidant agent.

The anticancer property of *P. aurata* was evaluated by cytotoxicity test against P388 murine leukemia cancer cells. There are 4 categories of cytotoxicity according to U.S. National Cancer Institute, namely non-toxic if the IC_{50} value is > 500 µg/mL, weak cytotoxic if the IC_{50} value is 201-500 µg/mL, moderate cytotoxic if the IC_{50} is 21-200 µg/mL and very toxic if it has IC_{50} value < 20 µg/mL (Sajjadi et al., 2015; Alabsi et al., 2016; Amaani and Dwira, 2018; Widiyastuti et al., 2019). This bioassay gave IC_{50} value of 0.08 mg/mL or equal to 80 µg/mL, it means the *P. aurata* crude extract had moderate cytotoxicity against P388 murine leukemia cancer cell. It is suggested that the presence of alkaloid compounds in the crude extract has correlation to the anticancer activity. Plenty of alkaloid-derivative compounds were widely reported as the anticancer agent from marine tunicate (Cooper and Yao, 2012; Palanisamy et al., 2017; Arumugam et al., 2020). Previous studies successfully discovered several cytotoxic compounds from *P. aurata* such as polycarpaurines A-C and polycarpathiamines (Wang et al., 2007; Pham et al., 2013). Interestingly, isolation of new cytotoxic compounds from *P. aurata* is less reported; therefore, this preliminary study gives an additional data on its prospect as an anticancer agent (Cooper and Yao, 2012; Arumugam et al., 2017, 2020; Khalifa et al., 2019).

5. Conclusion

Marine *P. aurata* from Indonesia exhibited antibacterial activity against MDR *B. cereus*, MRSA, *S. typhi* and non-MDR *E. coli*, while the antifungal assay indicated the absence of bioactivity. The crude extract showed very weak antioxidant activity with 534.60 ppm and a moderate cytotoxic against P388 murine leukemia cancer cell with IC_{50} value of 0.08 µg/mL. Phytochemical test indicated the presence of alkaloid and steroid/triterpenoid. The HPLC chromatogram gave six major peaks in the crude extract.

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Analysis of Phytochemical Constituents by using LC-MS, Antifungal and Allelopathic Activities of Leaves Extracts of *Aloe vera*

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Abstract

This study was conducted to investigate the phytochemical compounds of *Aloe vera* leaves by using LC-MS and evaluate their antifungal and allelopathic activities *in vitro.* The ethanol extract of *Aloe vera* leaves was subjected to evaluate their antifungal activity against three plants pathogenic fungi, *Fusarium roseum*, *Fusarium oxysporum lycopersici* and *Botrytis cinerea* by using the agar plate diffusion plate method. The aqueous extract of *Aloe vera* leaves was subjected to evaluate the allelopathic potential on germination and seedling growth of durum wheat *Triticum durum* and *Amaranthus hybridus* which is an advantice species of cereals. We used the bioassay of germination and seedling growth of both species. By LC-MS analysis, 11 bioactive phytochemical compounds were identified in ethanol extract:5-((S-2'-oxo-4'-hydroxypentyl-2(ß-glucopyranosyl-oxy-methyl)chromone, isoaloerisin D, aloenin, aloeninB, aloenin-2`-p-coumaroyl ester, aloe-emodin-diglucoside, 10-hydroxyaloin B, 10-hydroxyaloin A, aloin B, aloin A, aloveroside B. The antifungal activity showed that the ethanol extract *has an inhibitory activity against all the mycelial strains.* The allelopathic effect of different concentrations slowed the kinetics of germination of *Triticum durum* and *Amaranthus hybridus* and significantly decreased the rate of final germination of *Amaranthus hybridus* (100% at C10% and C25%), and a weak effect of inhibition of the germination was noticed for *Triticum durum* (40% at C25%). The aqueous extract has an inhibitory effect on the length of the roots (98% at C25%) and the height of the stems (100% at C25%) of *Triticum durum.* All concentrations of the aqueous extract have an inhibitory effect on the root length of *Amaranthus hybridus.* These results showed that *Aloe vera* would be suggested as a new potential source of natural herbicides and fungicides.

Keywords: *Aloe vera,* allelopathic potential, antifungal activity, extract, LC-MS.

1. Introduction

Aloe vera is a medicinal and ornamental plant belonging to *Lilaceae* family, usually originating in the dry regions of Africa, Asia, and Southern Europe, especially in the Mediterranean regions (Urch, 1999; Rodr´iguez *et al.* 2010). It is being cultivated in other areas with different climatic conditions (Rodr´iguez *et al.* 2010). Mexico is the main producer of *Aloe vera*, followed by Latin America, China, Thailand, and the United States (Rodr´iguez *et al.* 2010). Vitamins, enzymes, minerals, starch, lignin, anthraquinones, saponins, salicylic acid, and amino acids are among the more than 75 nutrients and 200 active compounds contained in *Aloe vera* (Park and Jo, 2006).

Previous research has revealed that phenolics such as chromone, anthrone, and phenyl pyrone are the most common secondary metabolites found in Aloe species' exudates. It is noteworthy that C-glycosylated chromones are found to represent a class of naturally occurring secondary metabolites that are known to be unique compounds in aloe, not having been reported in other plants (Franz and Grün, 1983).

This plant has several biological properties: anti-inflammatory (Afzal *et al*., 1991; Malterud *et al*., 1993), immuno-stimulatory (Ramamoorthy and Tizard, 1998), antiviral (Khalon *et al.,* 1991), cell growth stimulatory activity (Tizard *et al*., 1994), and antifungal (Kawai *et al.,* 1998).

Fungal diseases of crops are usually controlled using resistant cultivars, fumigants and long rotations, but mainly by using fungicides (Rongai *et al.,* 2015). The widespread use of fungicides to combat plant diseases has resulted in the accumulation of toxins in both humans and the environment. (Cherkupally *et al.,* 2017).

In an effort to minimize the use of synthetic fungicides, alternative methods to combat fungal diseases have been investigated using compounds obtained from plant sources. More than 500 plant species have recently been tested for antifungal activity. (Rongai *et al.*, 2012). Just

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3% of the plants studied had significant antifungal activity. Many authors have also studied the importance of secondary metabolites in antifungal activity (Rongai *et al.*, 2015). The relationship between antifungal activity and total phenolic content has also been reported (Stanković *et al.,* 2012; El-Khateeb *et al.,*2013).

The presence of weeds in a cereal field can be harmful in many ways. The phenomena of competition between weeds and crops, in particular, contribute to yield losses (Le Bourgeois and Merlier, 1995) by directly affecting crop growth. To control these weeds, massive quantities of chemical herbicides are used around the world (Turk and Tawaha, 2003). Synthetic herbicides, on the other hand, are often harmful and trigger environmental issues (Sharifi Rad *et al*., 2014; Khanh *et al.,* 2004; Sodaeizadeh *et al*., 2009). Mechanical weeding methods used in biological agriculture offer an alternative (Mason and Spanner, 2006; Bond and Grundy, 2001; Jordan, 1993), but their limit is linked to energy costs and working time for the farmer. Allelopathy is considered a promising technique for biological control (Ravlić *et al.,* 2017; Lovett, 1991). Allelopathy is a biological process in which plants interact with one another, either negatively or positively, by releasing allelochemicals into the environment. (Ravlić *et al.,* 2017; Rice, 1984). Allelochemicals are found in the stems, leaves, seeds, inflorescences, fruits, roots, and seeds of plants as end products, by-products, and metabolites (Sisodia and Siddiqui, 2010).

Other organisms, such as plants, animals, and microorganisms, are affected by the release of these chemical compounds into the atmosphere, and their behavior is either inhibited or stimulated (Fujii et al., 2003). There is mounting evidence that these plant chemicals can inhibit weed species' germination and development (Mohsenzadeh *et al*., 2011; Singh *et al.,* 2003; Turk and Tawaha, 2003; Sampietro and Vattuone, 2006).

Allelopathy's role in the natural management of weeds and crop growth is now widely acknowledged. (Sharifi Rad *et al.,* 2014; Mohsenzadeh *et al.,* 2010; Khan *et al*., 2009). In recent years, medicinal plants have been increasingly explored for their allelopathic potential (Modallal and Al-Charchafchi , 2006; Anjum *et al.,* 2010).

The present study aims to investigate the *Aloe vera* phytochemical compounds by using LC-MS and to evaluate their antifungal and allelopathic activities *in vitro.*

2. Materials and Methods

2.1. Chemicals and Reagents

Ethanol, methanol, acetonitrile, DMSO and water were obtained from Sigma Chemical Co (Sigma-Aldrich GmbH, Stern-heim, Germany). Formic acid was obtained from Biochem Chemopharma.

2.2. Plant materials

The leaves of *Aloe vera* were collected in October 2016 from the *Aloe vera* farm in Almeria (Spain). Then, the leaves of the plant were cleaned, dried and ground into powder.

Triticum durum and *Amaranthus hybridus* seeds were collected in El Tarf (Algeria) and stored in paper bags.

2.3. Fungal agents

Three plant pathogenic strains, *Fusarium roseum*, *Fusarium oxysporum lycopersici* and *Botrytis cinerea* were obtained from Biotechnology Research Center (CRBT), Laboratory of Mycology, Constantine, Algeria.

2.4. Preparation of ethanol extract

10 g of leaf powder was mixed with 100 mL of ethanol/water (80:20, v/v) for 24 h at room temperature. The extraction was performed 3 times with renewal of the solvent. A double-layered muslin cloth was used to filter the contents, followed by Whatman filter paper n°1. The bacteria were then removed using a micro-filter with a pore size of 0.22 µm. The filtrate was evaporated to dryness using a rotary evaporation at 40° C to yield 19% of the dry weight of residue which was stored at 4° C until use. The extraction yield was calculated and expressed as the percentage of the extract in relation to the mass of crushed leaves (%, w/w).

2.5. Qualitative determination of the phenolic contents of Aloe vera leaves

Qualitative analysis of phenolic compounds in the ethanol extract of *Aloe vera* leaves was carried out according to the protocol described by Bidel *et al.*(2015). 5mg of ethanol extract was mixed with 1mL of the methanol-water mixture (50/50, v/v). The sample was then ultrafiltered (10 min at 1000 rpm on Eppendorf centrifuge). 10 µL of the supernatant solution were injected into the chromatography chain via its automatic injector.

Chromatographic separation was carried out on a XTerra MS equipped with a binary HPLC pump (Waters 1525l, Waters, Manchester, UK), ESCi multimode ionization mass spectrometer (Micromass Ltd, Manchester, UK) equipped with an electrospray ionization ion source (ZSpray MKII) and a Waters 996 photodiode array detector. The tested extract was separated on an Agilent C_{18} column (3.5 µm particle size, 2.1×100 mm) at 40° C. A flow rate of 210 µL/min was used. Mobile phases consisted of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1 % (v/v) formic acid to minimize the ionization of phenolics (around pH 3.0). The capillary voltage was 2.5 kV. Nitrogen was used as the desolation gas (400 L/h) and cone gas (50 L/h). In the first step, spectra were reported in both negative and positive modes in full scan mode over the m/z 50-1200 range. Absorbance spectra in the 210-800 nm range. Mass spectra and absorbance were handled using MassLynx 3.5 software (Micromass Ltd).

2.6. Antifungal activity

The antifungal activity of *Aloe vera* leaves against three plant pathogenic fungi (*Fusarium roseum*, *Fusarium oxysporum lycopersici* and *Botrytis cinerea)* was tested using the agar plate diffusion plate method.

Six dilutions of the ethanol extract were prepared in DMSO (dimethylsulfoxide) (0.15%, 0.31%, 0.62%, 1.25%, 2.5% and 5%). 1 mL of each concentration was added to 15 mL of PDA culture medium (Potato dextrose agar). After solidification of the medium, 5 mm diameter mycelial discs from the margin of seven days old fungal cultures were placed in the center of each petri plate. (1disc/petri plate). Three replicates were performed for each extract concentration. Untreated medium (0%) was

used as control. The petri plates were incubated for 144 hours in the oven at a temperature of 20± 2°C.

Fungal growth was measured by averaging the two perpendicular diameters of each growing colony. The following formula was used to calculate the growth inhibition percentage (%):

Growth inhibition % = [(dc - dt)/dc] × 100

Where dc is the colony diameter in control plates and dt is the colony diameter in treated plates.

The minimum inhibitory concentrations (MIC) of the effective plant extract is defined as the lowest concentration of the antifungal agent that inhibits the fungal growth.

2.7. Allelopathic potential

2.7.1. Preparation of concentrations

Different concentrations (1%, 5%, 10% and 25%) were prepared by maceration the powder from the leaves of *Aloe vera* under agitation in distilled water for 24 hours. The content was then filtered through Whatman filter paper n°1, and then the extract obtained was stored at 4°Cuntil use.

2.7.2. Test for inhibition of germination and growth of Amaranthus hybridus and Triticum durum

Amaranthus hybridus and *Triticum durum* seeds were arranged in petri plates (10 seeds/petri plate) with filter paper moistened with *Aloe vera* aqueous extract at various concentrations. The control was prepared with distilled water. Germination tests were conducted in an oven at a constant temperature of 25°C.

2.7.3. Observations and Measurement

Observations and measurements included the kinetics, seed germination rate and seedling growth (stem height, root length) of *Amaranthus hybridus* and *Triticum durum*. The notations were carried out daily for 8 days. Three repetitions were performed out for each concentration of the extract.

2.7.3.1. Determination of germination percentages

After 8 days of incubation, the germination percentage of each species is determined. Sprouted seeds are any seed that has developed a coleorhiz in monocotyledonous species or a radical in dicotyledonous species.

2.7.3.2. .Determination of inhibition percentages

Inhibition percentages were calculated according to the formula used by Dhima *et al*. (2006) and Chung *et al.* (2003):

Germination inhibition %= [(G-g) / G] × 100

Where G is the germination in the control (distilled water) and g is germination in different concentrations of the extract.]

Growth inhibition % = [(H-h) / H] × 100

Where H is stem height or root length in the control (distilled water) and h is stem height or root lengthin different concentrations of the extract.

2.8. Statistical analysis

All assays were estimated in triplicates and the results were presented as means ± standard deviation (SD). Statistical analysis was performed using Variance Analysis (ANOVA, SAS), and averages were compared according to the student test. The results are considered to be significant when $p < 0.05$.

3. Results

3.1. Qualitative determination of the phenolic contents of Aloe vera leaves

Chromatogram of ethanolic extract by LC-MS analysis was shown in Figure 1 and the characterized compounds were presented in Table 1, identified with the numbers 1-11 considering the elution order. The compounds were divided into four classes in this study: chromones, phenyl pyrones, naphthalene derivatives, and anthrones.

The determination of phenolic compounds classes found were identified using their UV spectra obtained by LC-MS data, and then confirmed by comparison with literature data.

Two peaks 2 and 3 from 5-((S-2'-oxo-4'-hydroxypentyl-2(ß-glucopyranosyl-oxy-methyl)chromone and isoaloerisin D, respectively) were identified as chromones. These compounds have been identified by Wu *et al.*(2013); Fanali *et al.* (2010). Three phenyl pyrones (peaks 4, 8 and 11) eluting at 23.2, 32.8 and 55 min, giving [M− H]− ions, were identified as aloenin, aloenin B and aloenin-2`-p-coumaroyl ester, respectively (Wu *et al*., 2013).

Five Peaks 1, 5, 6, 9 and 10 (from Aloe-emodin-diglucoside,10-Hydroxyaloin B, 10-Hydroxyaloin A, Aloin B, Aloin A, respectively) were characterized as anthrones. The results were in accordance with those reported by Wu *et al.*(2013); Fanali *et al.* (2010); Quispe *et al.*(2018). Peak 7 was characterized as aloveroside B (Naphthalene derivative), similar to previously reported aloveroside A (Wu *et al.,* 2013; Yang *et al.,* 2010).

Table 1. Peak assignments of metabolites identified by LC-MS of ethanol extract of *Aloe vera* leaves.

Peak number[a]	Retention time (min)	Molcular formula	MS[1] ion		Fragments MS[2]	Tentative Identification	References
1	14.97	$C_{27}H_{29}0_{15}$	593	[M-H]-	377	Aloe-emodin-diglucoside	(Quispe*etal*.,2018)
2	15.6	$C_{21}H_{26}O_{10}$	461	[M+H]+	443, 417, 399, 249, 185	5-((S-2'-oxo-4'-hydroxypentyl2(ß- glucopyranosyl-oxy-methyl)chromone	(Wu *et al.*, 2013)
3	21	$C_{29}H_{32}0_{11}$	557.13	[M+H]+	437,513,393, 291,247,217	isoaloerisin D	(Wu *et al.*, 2013; Fanali*etal.,* 2010)
4	23.2	$C_{19}H_{22}O_{10}$	409.11	[M−H]−	247, 203, 171	Aloenin	(Wu *et al.*, 2013)
5	23.9	$C_{21}H_{22}O_{10}$	433	[M−H]−	270	10-Hydroxyaloin B	(Wu *et al.*, 2013)
6	27.9	$C_{21}H_{22}O_{10}$	433	[M−H]−	270	10-Hydroxyaloin A	(Wu *et al.*, 2013)
7	29.5	$C_{30}H_{40}O_{17}$	695	[M+H]+	549, 335	Aloveroside B	(Wu *et al.*, 2013)
8	32.8	$C_{34}H_{38}O_{17}$	717	[M-H]-	247, 555	Aloenin B	(Wu *et al.*, 2013;Fanali *etal.,* 2010)
9	48.5	$C_{21}H_{22}O_{9}$	417	[M-H]-	297, 268, 239	Aloin B	Wu *et al.*, 2013 ;Fanali *etal.,* 2010 ;Quispe*etal*., 2018.)
10	53	$C_{21}H_{22}O_{9}$	417	[M-H]-	297, 268, 239	Aloin A	Wu *et al.*, 2013 ;Fanali *etal.,* 2010 ;Quispe*etal*., 2018.)
11	55	$C_{28}H_{28}O_{12}$	555	[M-H]-	247, 391, 307	Aloenin-2`-p-coumaroyl ester	(Wu *et al.*, 2013)

[a]: The notation for peak number refers to figure 1.

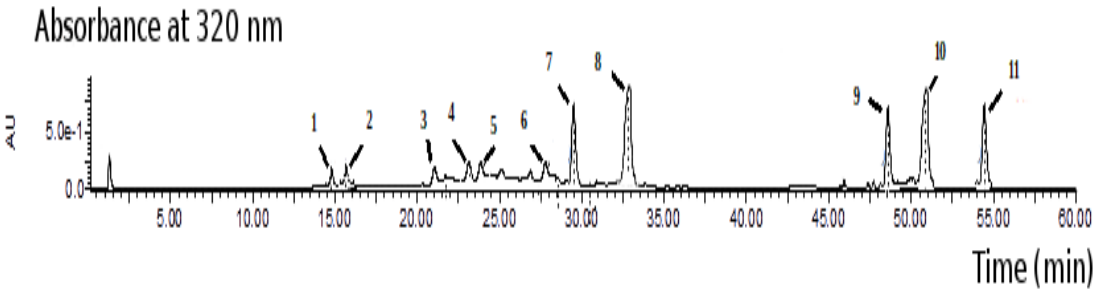

Figure 1. LC-MS chromatogram of ethanol extract of *Aloe vera* leaves at 320 nm expressed in absorbance units (AU).

3.2. Antifungal activity of Aloe vera

Antifungal activity of ethanol extract was determined against three plant pathogenic fungi, Fusarium roseum, Fusarium oxysporum lycopersici and Botrytis cinerea. The results of the *Growth inhibition percentage* of *ethanol extract* show that the ethanol extract has an inhibitory activity against the tested mycelial strains (Figures 2 and 3).

It is noted that the ethanol extract at concentrations (5%, 2.50%, 1.25% and 0.62%) partially inhibited the growth of *Fusarium roseum* with an inhibition rate (92%±2.64, 85%±3.60, 77.33%±1.15 and 51%±2.64, respectively), and at the concentrations (5% and 2.50%) partially inhibited the growth of *Fusarium oxysporum lycopersici* with an inhibition rate (71%± 4.58and 55%±1.63).

Results showed that the ethanol extract at the concentrations (5%, 2.50% and 1.25%) totally inhibited the growth of *Botrytis cinerea* (100%±0.00) and MIC of about 1.25%. The ethanol extract at concentrations (0.62% and 0.31%) partially inhibited the growth of this plant pathogenic fungi with an inhibition rate (72%±2.16 and 63.67%±0.57, respectively).

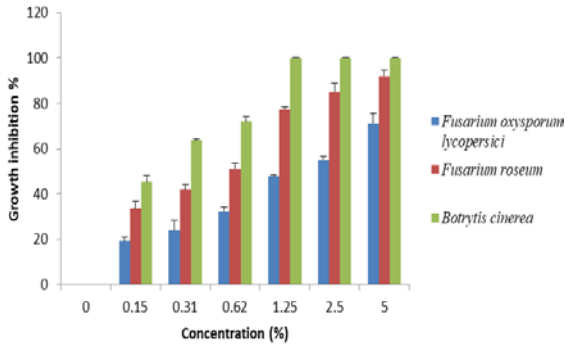

Figure 2. Growth inhibition percentage *of* ethanol extract concentrations *of Aloe vera* leaves.

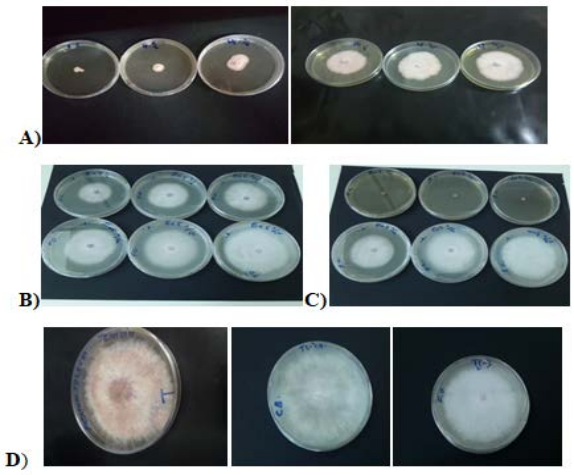

Figure 3. *Fusarium roseum* (A), *Fusarium oxysporum lycopersici* (B) and *Botrytis cinerea (C)* colonies grown on PDA medium supplemented with ethanol extract of *Aloe vera* leaves, tested at six concentrations (5%, 2.5%,1.25%, 0.62%, 0.31% and 0.15%,) recorded after 144 hours of incubation at 20°C. Negative control (D): Untreated control (PDA + DMSO).

3.3. Allelopathic potential

3.3.1. Effects of the aqueous extract of Aloe vera on germination of Triticum durum and Amaranthus hybridus

Different concentrations of the aqueous extract of *Aloe vera* leaves slow the germination kinetics of *Triticum durum* and *Amaranthus hybridus* seeds (Figures 4 and 5) and significantly reduce the rate of final germination of *Amaranthus hybridus*.

The germination inhibition effect of *Amaranthus hybridus* reaches a maximum value of 100% for C10% and C25%. However, a weaker germination inhibition effect (40%) was observed for *Triticum durum* at the concentration C25%.

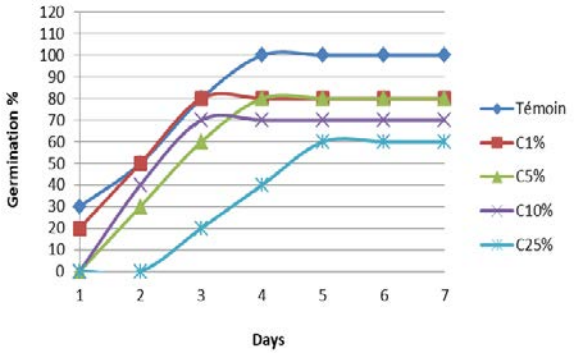

Figure 4. Effect of the aqueous extract of *Aloe vera* leaves on the germination kinetics of *Triticum durum.*

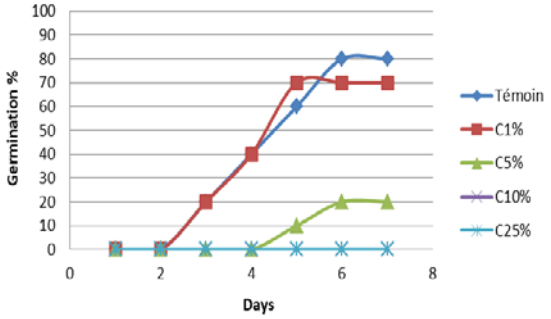

Figure 5. Effect of the aqueous extract of *Aloe vera* leaves on germination kinetics of *Amaranthus hybridus*

Most of the studied concentrations of aqueous extract have an important inhibitory effect on root length and stem height of *Triticum durum* and *Amaranthus hybridus* seedlings (Table 2). The aqueous extract has an inhibitory effect on the length of the roots and the height of the stems of *Triticum durum* from the concentration C5% and reaches a maximum value at the concentration C25% (98.08%, 96.97% for roots and stems). At the C1% concentration, however, the aqueous extract does not represent an inhibitory effect on the root length and height of *Triticum durum* stems, which is respectively 7.20± 0.16cm, 4.4± 0.96cm.

The aqueous extract from the leaves of *Aloe vera* has an inhibitory effect on the length of the roots of *Amaranthus hybridus* at all concentrations and completely inhibits root growth (100%) at C10% and C25% concentrations.

Table 2. Effect of the aqueous extract of *Aloe vera* leaves on the growth of the roots and stems of *Triticum durum* and *Amaranthus hybridus.*

Concentrations	Length of *Triticum durum roots*		Height of the stems of *Triticum durum*		Length of *Amaranthus hybridus* roots	
	cm	Inhibition%	cm	Inhibition%	cm	Inhibition%
Control	7.8±0.28^{a}	0.0±0.00	5.5 ±0.70^{a}	0.0±0.00	5.16±0.62^{a}	0.0±0.00
C1%	7.20±0.16^{a}	8.05±1.41	4.4±0.96^{a}	23.02±3	1.8 ±0.28^{b}	64.44±3.84
C5%	1.83±0.28^{b}	75.97±4.57	0.63±0.15^{b}	88.56±1.25	0.16±0.05^{c}	93.84±4.31
C10%	1.7±0.36^{b}	79.13±4.39	0.16±0.05^{c}	90.1±2.15	0.00±0.00^{d}	100±0.00
C25%	0.15±0.05^{c}	98.08±0.62	0.1±0.05^{c}	96.97±1.00	0.00±0.00^{d}	100±0.00

In the same column, the means followed by the same letter are not significantly different according to the student test at $p<0.05$. Each value represents a mean standard deviation (n=3).

4. Discussion

Evaluation of the antifungal potential of *Aloe vera* leaves in our study is consistent with previous work reports. It should be noted that Casian *et al*. (2007) showed that the *Aloe vera* leaf extract inhibit the mycelial growth of *Penicillium gladioli, Fusarium oxysporum, Heterosporium pruneti* and *Botrytis gladiolorum.* Jasso *et al*. (2005) assessed the antifungal potential *of Aloe vera* leaf extract against the mycelium development of *Rhizoctonia solani, Fusarium oxysporum* and *Collectotrichum coccodes* and found positive results. Saks and Barkai-Golan (1995) tested the antifungal potential of *Aloe vera* leaf on four plant pathogenic fungi, *Botrytis cinerea*, *Alternaria alternata, Penicillium digitatum* and *penicillium expansum;* the result of this study showed that the *Aloe vera* leaves suppress the mycelial growth of all mycelial strains tested. In our study, the *Aloe vera* leaves completely inhibited the growth of *Botrytis cinerea* (100%). However, Saks and Barkai-Golan (1995) showed that *Aloe vera* leaves partially inhibited the growth of *Botrytis cinerea* (67%), The difference between these results may be due to geographical and climatic conditions, which may affect the phytochemical composition of the plant and its antifungal activity (Jeyasakthy e*t al*., 2017).

Bajwa and Shafique (2007) used *Aloe vera* extract against plant pathogenic strains, *Alternaria alternata, Alternaria citri* and *Alternaria tenuissima*. The results of this study reflect that *Aloe vera* has an inhibitory effect on mycelial growth and the proliferation of these fungi.

Cooposamy and Magwa (2007) also proved that *Aloe vera* extract had an antifungal effect on *A. flavus, A. glaucus, C. albicans, C. tropicalis, T. mentagrophytes* and *T. rubrun*. The study by Sitara *et al.*(2011) showed that *Aloe vera* gel extract completely inhibited the growth of *Drechslera hawaiensis* and *Alternaria alternata*.

Eugene *et al.* (2011) showed that aloin and aloe-emodin have a significant inhibitory effect on the growth of *C.gloeosporioides* and *C.cucumerinum*. Aloin and aloe-emodin are anthraquinone derivatives, and antifungal activity of several anthraquinone derivatives in other plants has been reported by Agarwal *et al.*(2000); Singh *et al.* (2006). These studies were consistent with our study.

Results obtained on seed germination and development of the seedlings of *Triticum durum* and *Amaranthus hybridus* show that the aqueous extract of *Aloe vera* delays seed germination to the point of interruption.

Kruse *et al*. (2000) showed that when susceptible plants are exposed to allelochimical substances, seed germination is delayed. For some seeds, germination stops in the swelling stage of the seed. For others, germination stops at the beginning of the radicle's appearance.

When seed germination is not inhibited, other effects on seedling development have been observed. In the case of inhibition, we noted effects on the root and stem or both. In some cases, the root development is delayed; in other cases, the root development stops. For the aerial part, the effect is manifested by the delay of development or by the absence of stem.

In most of the tests carried out in our study, the aqueous extract's inhibitory effect is more important on the growth of the seedlings (root length and aerial length) and increases when the concentration of the extract increases, Arslan *et al.* (2005), Uremis *et al.* (2005), Turk and Tawaha (2003) and Batish *et al.* (2002), Baličević *et al.* (2014) also showed that inhibition increases with increased concentration of extracts.

Seed germination of the adventitious species *Amaranthus hybridus* is completely inhibited by *Aloe vera* extract at C10% and C25% concentrations. We also tested a variety of wheat to see if the allelopathic plant also affects wheat crops or not. Based on the results obtained, the aqueous extract has no effect on wheat seed germination. However, the aqueous extract affects the development of the roots of the species *Triticum durum* at concentrations C5%, C10% and C25%. These results were in agreement with those of Hussain *et al.* (2007), which showed that the extract (C10%) of *Cassia angustifolia* inhibits root length but does not affect wheat seed germination. Another study by Alipoor *et al* (2012) showed that *Aloe vera* leaf extract exhibited a significant inhibitory effect on the seed germination and seedling lengths of *Triticum aestivum, Secale cereale* , *Lepidium sativum, Amaranthus retroflexus, Taraxacum officinalis.* Wheat seeds *(Triticum aestivum)* were least affected by the *Aloe vera* extract. Seed germination and seedling growth of *Lepidium sativum, Amaranthus retroflexus,* and *Taraxacum officinalis* were almost completely inhibited at 2.5% of leaf extract.

In our study, seed germination of the adventitious species, *Amaranthus hybridus*, is completely inhibited by the aqueous extract at concentrations C10% and C25%. However, other studies have reported negative effects of aqueous extract from *Aloe vera* leaves on crops such as the results observed by Lin *et al*. (2004); they reported a negligible effect on germination of *Lactuca sativa*, *Raphanus raphanistrum*, *sativus, Brassica rapa* and *Oryza sativa.* Also, the study by Ravlić *et al.* (2017) showed that the aqueous extract from the leaves of *Aloe vera* does not affect germination of *Amaranthus retroflexus L, Tripleurospermum inodorum L, Abutilon theophrasti.*

The inhibitory effect of *Aloe vera* leaf extract on seed germination and seedling development may be related to the presence of allelochimic compounds, including tannins, flavonoids and phenolic acids. In addition, toxicity may be caused by a synergistic effect of several secondary metabolites (Saharkhiz *et al.* 2009; Nourimand *et al.* 2011).

5. Conclusion

In this study, the analysis of Phytochemical Constituents attested to the presence of chromones, phenyl pyrones, naphthalene derivative, and anthrones in ethanol extract of *Aloe vera* leaves. Our results bring new information to the literature data about the antifungal activity of *Aloe vera* extract against *Botrytis cinerea;* the ethanol extract at the concentrations (5%, 2.50% and 1.25%) completely inhibited the growth of these mycelial strains. The aqueous extract of *Aloe vera* inhibits the germination of a wheat weed species (*Amaranthus hybridus*) without affecting the germination of durum wheat. Total germination inhibition of *Amaranthus hybridus* is noted at C10% and C25%.

The isolation and purification of several polyphenolic components of *Aloe vera* are necessary for the development of natural derived herbicides and fungicides. Their use would minimize the use of synthetic herbicides and fungicides over time, which would be a significant economic benefit to farmers and would also reduce adverse impacts on the environment.

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Anisakis Infection of Belanger's Croaker (*Johnius Belangerii* Cuvier 1830) at The Indian Ocean Coast of Yogyakarta, Indonesia

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Abstract

This research intended to find out the prevalence, intensity, and species of *Anisakis* (Nematoda) larvae infecting Belanger's croaker (*Johnius belangerii*) at Indian Ocean coast of Yogyakarta. Totally, 150 samples of Belanger's croaker fish collected from fishermen were used for this experiment. After measuring the total length and weight, each sample was examined for *Anisakis* larvae infection in the body cavity, internal organ, and muscle. Results showed that Belanger's croaker was susceptible to *Anisakis* infection by moderate prevalence (48.7%) with a mean intensity of 5.7 larvae/individual. All *Anisakis* larvae were found in the abdominal cavity (100%). Morphological identification by Scanning Electron Microscopy showed that the *Anisakis* larvae infecting the Belanger's croaker was *Anisakis* Type I, marked by a boring tooth on the anterior part and a mucron at the caudal end. Molecular identification applying PCR-RFLP of the ITS region, sequencing, and phylogenetic analysis of the mitochondrial DNA cox2 gene confirmed the larvae as *Anisakis typica* var. *indonesiensis*. The presence of *A. typica* in Belanger's croaker is estimated to pose a small risk to human health. *Anisakis typica* is known as not zoonotic *Anisakis* species. Moreover, the infection occurred in a relatively moderate prevalence, low intensity, and larvae were not found at the edible part of fish. From another point of view, these larvae are considered as useful biological indicators for several ecological parameters and further studies.

Keywords: Infection Rates, Prevalence, Zoonosis, Human Health Risk, Food Safety

1. Introduction

The genus *Anisakis* (Nematoda, Family Anisakidae) is strictly parasitic, infecting various marine organisms. These parasites are cosmopolitans and have a wide distribution, although the species are found in limited, partly overlapping, areas (Kuhn *et al*., 2013). *Anisakis* has been studied because of its zoonotic effect on human health, impact to the fisheries industries economy (Pozio, 2013), and was subject of ecological studies, e.g. for fish stock separation (Palm, 2011). *Anisakis* spp. are potentially zoonotic endoparasites; several species are known to cause the anisakiasis, a disease that is transmitted from fish to humans (Sakanari and Mc Kerrow, 1989). The 3rd-stage larvae of *Anisakis* spp. are transmitted to humans consuming raw or uncooked infected fish. Symptoms of *Anisakis* infection in humans include vomiting, nausea, diarrhea, or allergic reactions (Adroher and Benitez, 2020). Ecological studies used *Anisakis* as biological tags of fish migration and movement pattern, stock discrimination, recruitment, reproduction, and for food web structure analyses (MacKenzie, 2002; Podolska *et al*., 2006; Palm, 2011).

Nowadays, various fish species and cephalopods are infected with *Anisakis* larvae. *Anisakis* on fish/cephalopods have been well studied in Europe and America, and e.g. parts of Asia (Kuhn *et al*., 2013). Many pelagic and demersal fish species with high economic value, such as scad (*Decapterus* spp.), frigate tuna (*Auxis thazard*), mackerel (*Scomber* spp.), skipjack tuna (*Katsuwonus pelamis*), hairtail fish (*Trichiurus* spp.), and jack/trevallies (*Caranx* spp.), are potential hosts and have been reported infected with *Anisakis*. Several studies related to local Indonesian *Anisakis* species and infection patterns have been conducted (Hutomo *et al*., 1978; Setyobudi *et al*., 2007; Suadi *et al*., 2007; Palm *et al*., 2008; Setyobudi *et al*., 2011a; Anshary *et al*., 2014; Palm *et al*., 2017, Theisen, 2019), but the number of known local fish hosts is still relatively small when compared with the total number of marine fish species in Indonesian waters. There are 6 genotypes of *Anisakis* found in Indonesian and adjacent waters. Among these nematodes, *Anisakis typica* is the most common, and there are even some local genotypes that may differ from the genotypes that have been published so far. Therefore, in order to avoid identification confusion in the future, the Indonesian *A. typica* genotype was noted as the sub-specific entity namely *A. typica* var. *indonesiensis* (Palm *et al.*, 2017). **S**imilar result was shown of *A. typica* T isolated from *Priacanthus tayenus* in Gulf Thailand, and that local

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genotype was considered not a variety but a distinct species status (Eamsobhana *et al*. 2018).

Belanger's croaker is a demersal fish, not yet reported to be infected with *Anisakis* in Indonesia (compare host-parasite checklist for Indonesia in Theisen-2019), even though several studies reported *Anisakis* infection was found on other demersal fish. Interestingly, *Anisakis* infections of Indonesian croakers (Sciaenidae) seem to be rare generally as listed in the local host-parasite checklist for fish parasites (Theisen 2019). Therefore, the detected presence of *Anisakis* spp. on Belanger's croaker makes this fish species a promising biological indicator for subsequent ecological studies (MacKenzie, 2002; Mattiucci *et al*., 2008, Palm 2011; Munster 2015), and can help to improve handling and processing of this consumable fish. The presence of *Anisakis* in marine fish can also be used as an indicator for fish quality. *Anisakis* infection in fish can cause aesthetic deterioration and reduce product value (Aspholm, 1995). Thus, fish with massive infections are regularly avoided by salesmen and consumers, consequently with economical loss, but also preventing human anisakiasis (McClelland, 2002).

Anisakis nematodes can be identified morphologically and molecularly. However, the larvae in fish are morphologically difficult to distinguish. Compared with morphological identification, molecular identification is more efficient and accurate and can overcome its limitations, because reference DNA regions for every valid *Anisakis* species are known and available (Mattiucci and Nascetti, 2008). Molecular techniques that can accurately identify the species of *Anisakis* have been developed rapidly and widely used, such as Polymerase Chain Reaction-Restricted Fragment Length Polymorphism (PCR-RLFP) and direct sequencing (D'Amelio *et al*., 2000; Nadler *et al*., 2005). PCR-RFLP of the Internal Transcribed Spacer (ITS) and 5.8S ribosomal DNA (rDNA) regions can be applied for *Anisakis* species identification (Umehara *et al*., 2006; Anshary *et al*., 2014). However, PCR-RFLP method cannot be used to study in detail the genetic variation of both intra and inter species. Furthermore, ITS region and mitochondrial DNA (mtDNA) cox2 gene analyses have been used to determine the genetic relationship and genetic variation between *Anisakis* species (Farjallah *et al*., 2008; Umehara *et al*., 2010; Eamsobhana *et al*. 2018). Recently, Mattiucci et al. (2017) reviewed and discussed taxonomical aspects related to biodiversity assessment, emphasizing on fish parasitic worm taxa recognized as biological species based on molecular/genetic markers.

2. Material and Methods

2.1. Fish Sampling and Anisakis Larvae Collection

This study sampled 150 Belanger's croaker fish (*Johnius belangerii*) (107 male and 43 female) caught by artisanal fishermen during February–August 2019 from the coast of Yogyakarta, Indonesia (Indian Ocean) (7°53′47″-8°11′56″ SL; 110°17′32″-110°47′32″ EL). The samples were identified based on Carpenter and Niem (2001), then measured for its total length using meter scale (accuracy 0.1 cm) and weight using electronic balance (accuracy 0.1 g), dissected, then observed for *Anisakis* at the abdominal cavity, liver, digestive tract, gonads, and muscle. The collected *Anisakis* were immersed and washed in 0.9% solution of sodium chloride and water, then stored in absolute ethanol for subsequent analysis (Setyobudi *et al*., 2019). The infection parameters, i.e. prevalence (P) and mean intensity (MI), were determined following Bush *et al*. (1997).

2.2. Anisakid Identification

2.2.1. Scanning Electron Microscopy (SEM)

Anisakis larvae were processed and cleaned at 4°C using cacodylate buffer for 6 h, prefixed using 2.5% glutaraldehyde for 12 hours, and then fixed with 2% tannic acid for 6 hours. After fixation process, the samples were washed in cacodylate buffer and gradually dehydrated in 30, 50, 70, 85, 90, and 99.6% ethanol. Specimens were positioned on the stub accordingly, and then covered with Au using Ion Coater SPT-20. The morphological characters of *Anisakis* were observed via a Hitachi SU-3500 SEM.

2.2.2. Molecular Identification

Twenty *Anisakis* larvae individuals were randomly selected for molecular identification. The DNA was extracted using Tissue/Blood DNA minikit extraction (Geneaid) by adhering to the manufacturer's procedure. The genomic DNA was used for further molecular analysis.

2.2.2.1. PCR-RFLP Analysis

The ITS region of rDNA (ITS-1, 5.8 S and ITS-2) of n = 20 worms was amplified using primer A (5′-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA-3′) and primer B (5′-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT-3′) (D'Amelio *et al*. 2000). The PCR conditions were: 94°C for 10 min for denaturation; 35 cycles of 94°C for 40 s, 54°C for 40 s, and 72°C for 90 s; and post amplification at 72°C for 7 min. Amplification products were analyzed by RFLP using *Taq*I, *Hha*I, and *Hinf*I DNA endonuclease restriction enzymes (D'Amelio *et al*. 2000). The digestion was carried out using 0.5 µL of endonuclease restriction enzyme, 1 µL of buffer, 3 µL of PCR product and 5.5 µL distilled water. Digestion with the *Hinf*I and *Hha*I enzymes was carried out at 37°C for 90 min, while digestion with the *Taq*I enzyme was carried out at 65°C for 90 min. The PCR-RFLP products were analyzed with 1.5% agarose gel electrophoresis with fluorosafe stain. The observed bands were used to determine the species following the previous research (D'Amelio *et al*., 2000; Anshary *et al*., 2014).

2.2.2.2. mtDNA cox2 Gene Sequencing

Polymerase Chain Reaction of the mitochondrial DNA cox2 gene (n = 4 worms) was conducted using primer 210 (5′-CAC CAA CTC TTA AAA TTA TC-3′) and primer 211 (5′-TTT CTA GTT ATA TAG ATT GRT TYA T-3′ (Nadler and Hudspeth 2000). The amplification was conducted under the reaction conditions: 94°C for 5 min; 35 cycles of 94°C for 40 s, 42°C for 40 s, and 72°C for 75 s; then post amplification at 72°C for 7 min. Then, the product was sequenced through a DNA sequencing service company (1stBase Laboratory in Malaysia through PT Genetika Science Indonesia). The results of nucleotide sequences were verified using BioEdit 7.0.4 software. The nucleotide's alignment was conducted using MEGA X,

followed by the construction of a phylogenetic tree based on mitochondrial DNA cox2 gene nucleotide sequences using the maximum likelihood method and Tamura-Nei model (Kumar *et al.,* 2018).

3. Results

3.1. Anisakis Infection

Belanger's croaker (*Johnius belangerii*, family Sciaenidae) is an economically important fish commonly caught locally. This demersal fish is distributed throughout Indonesian waters, inhabits coastal waters and estuaries, and feeds mainly on penaeid prawn and polychaeta (Simanjutak and Raharjo, 2001). In total, 150 fish samples with body length 14.6–28.2 cm and weight 35.6-339 g were collected from the coast of Yogyakarta, Indonesia. This research showed that Belanger's croaker was susceptible to *Anisakis* larvae infection. *Anisakis* infections of *J. belangerii* at the coast of Yogyakarta were moderate in prevalence (P) and mean intensity (MI) (P= 48.7%, MI=5.7 larvae/fish individual). The increasing prevalence and intensity of *Anisakis* in relation to increasing fish length are shown in Figure 1.

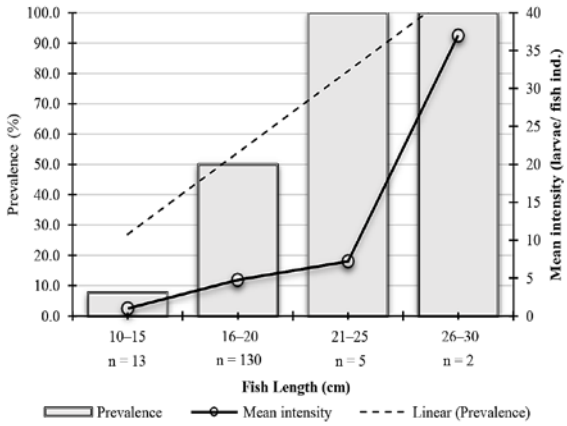

Figure 1. Infection rates of *Anisakis* on Belanger's croaker *Johnius belangerii* caught from the Indian Ocean coast of Yogyakarta

Figure 1 shows that the highest prevalence (100%) was observed in the fish with 21–25 cm length (W=109.7-121.8 g) (n=5), and 26–30 cm (W=282.0-339.0 g) (n=2), whereas the lowest prevalence (7.7%) was found in the fish with 10–15 cm body length (W=32.5-55.0 g) (n=13). The highest mean intensity of *Anisakis* infection was observed in the fish with 26–30 cm length (W=282.0-339.0 g) (37.0 larvae/fish individual), and the lowest was in the fish with 10–15 cm (W=32.5-55.0 g) (2.7 larvae/fish individual). These results indicate a relationship between the infection and body length. Both the prevalence and mean intensity of *Anisakis* infection tend to increase with increase in the body length. All *Anisakis* larvae were found freely or coiled the abdominal cavity and were not found in the other locations. The mean intensity distribution of *Anisakis* infection on Belanger's croaker is shown in Figure 2.

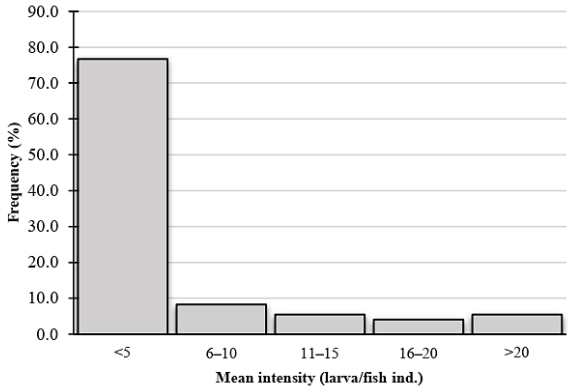

Figure 2. Distribution of intensity of *Anisakis* infection on Belanger's croaker *Johnius belangerii*

As shown in Figure 2, the collected Belanger's croakers were infected by *Anisakis* larvae in low intensity (<5 larvae/fish ind., 76.7 %), and only few were infected in high intensity (>20 larvae/fish ind., 5.5%).

3.2. Anisakis Identification

3.2.1. Scanning Electron Microscopy (SEM)

Morphological identification was carried out by analyzing four *Anisakis* samples via SEM (Plate 1).

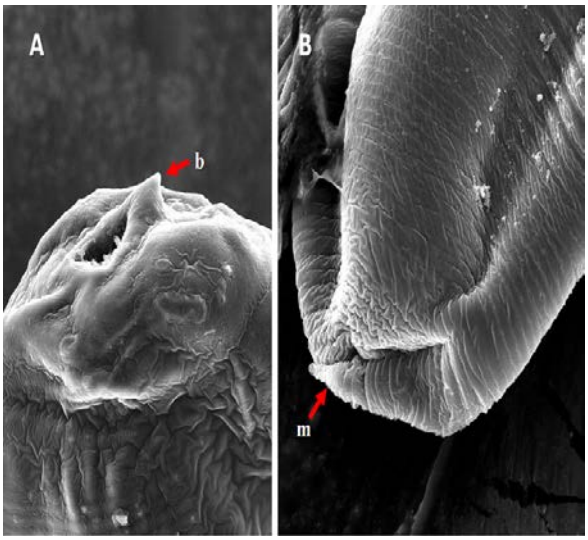

Plate 1. SEM Analysis (A: [b] = boring tooth; B: [m] = mucron) of Anisakis typica from Johnius belangerii

Figure 3 shows that *Anisakis* isolated from Belanger's croakers was Type I, marked by a boring tooth (b) on the anterior part and a mucron (m) on the posterior part.

3.2.2. Molecular Identification

3.2.2.1. PCR-RFLP Analyses

Amplification of the ITS region resulted in a band with a size of ~1 kb (Plate 2). The amplification product of the ITS region was then analyzed by RFLP. The results of digestion restriction enzyme are illustrated in Plate 3.

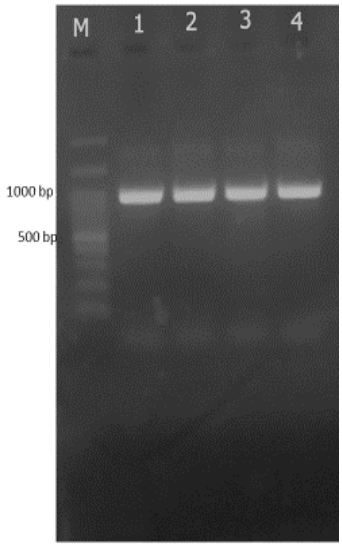

Plate 2. Electrophoresis visualization of the ITS rDNA region of *Anisakis typica* from *Johnius belangerii* (M=marker, 1-4: samples in this study)

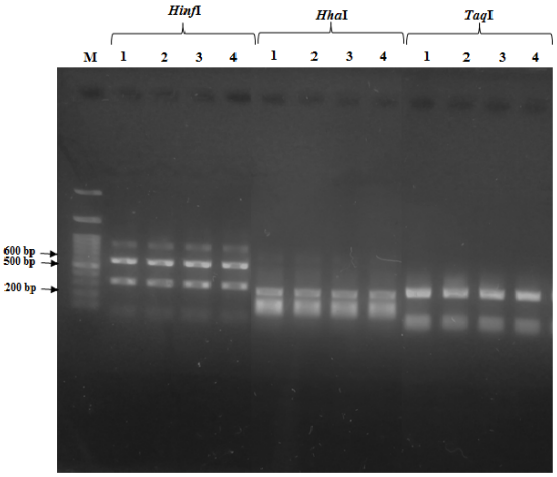

Plate 3. Visualization of digestion restriction enzymes of *Anisakis typica* from *Johnius belangerii* (M = marker, 1-4 = sample in this study)

Plate 3 shows the pattern after digestion of the PCR product using *Taq*I, *Hinf*I, and *Hha*I restriction enzymes. The digestion using *Taq*I formed two bands (400 base pairs (bp), 350 bp), *Hinf*I formed two bands (600 bp, 350 bp), and *Hha*I formed two bands (320 bp, 240 bp). All of the samples produce a similar pattern and corresponded to *Anisakis typica* (D'Amelio *et al*., 2000; Anshary *et al*., 2014).

3.2.2.2. Sequencing mtDNA cox2

PCR of the mtDNA cox2 gene showed band DNA with nucleotide length ± 600 bp. The electrophoresis results are shown in Plate 4.

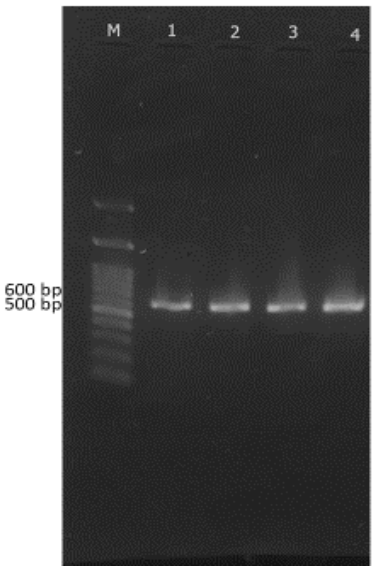

Plate 4. Visualization of *Anisakis typica* from *Johnius belangerii* electrophoresis with the mtDNA cox2 gene (M= marker, 1–4 = samples of this study).

Molecular identification using the mtDNA cox2 target gene confirmed that the *Anisakis* species infecting the Belanger's croaker was *A. typica* .

Anisakis infecting Belanger's croaker is similar *to A. typica* from the Philippines, the Persian Gulf, Thailand, Indonesia, Papua New Guinea, Egypt, Adriatic Sea, Brazil, and Japan. The phylogenetic tree constructed based on mitochondrial DNA cox2 genes sequences explains the genetic relationship of *A. typica* found in Belanger's croaker isolated from the coast of Yogyakarta with references from the NCBI GenBank (Figure 3). The phylogenetic tree shows that the *Anisakis* isolated from Belanger's croaker from Yogyakarta forming into two groups closely related to *A. typica* from the Philippines water, the Gulf of Thailand, the Papua New Guinea, and closely related to *A. typica* from the Persian Gulf.

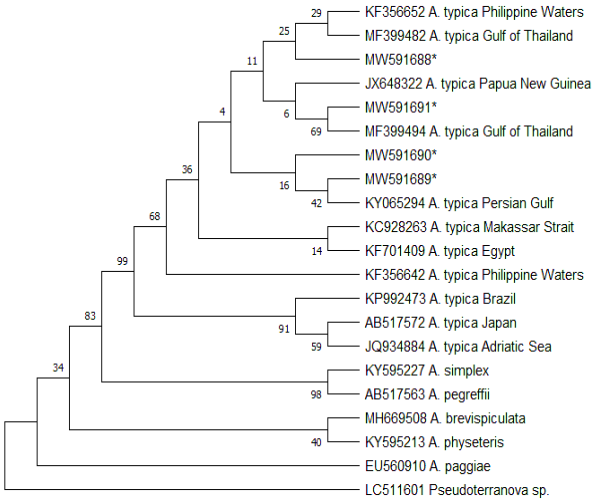

Figure 3. The molecular phylogenetic tree showing the genetic relationship among *Anisakis* species samples based on mtDNA cox2 genes. The phylogenetic tree was constructed using the maximum likelihood method according to the Tamura–Nei model (bootstrap=1000). The GenBank Accession numbers for the sequences from this study are: MW591688-MW591691. *= this study

4. Discussion

4.1. Locality and Host Records

Anisakis is a parasite infecting pelagic and demersal fish. Belanger's croaker *Johnius belangerii* is a paratenic host of *Anisakis* similar to other local demersal fish, such as *Trichiurus* spp., *Selaroides leptolepis*, and *Terapon jarbua* (Palm *et al*., 2017). It has never been reported to be infected in Indonesia (Theisen 2019). Belanger's croaker captured at the coast of Yogyakarta has a moderate prevalence and relatively high infection rate (P = 48.7%; MI = 5.7 larvae/fish individual). Previous studies reported variation in the prevalence, mean intensity and target organ of *Anisakis* larvae infection in marine demersal fish species. Setyobudi *et al*. (2011a) showed the differences of the prevalence of *Anisakis* infecting demersal fish caught at the coast of Yogyakarta; a high prevalence was found in Three-striped tiger fish (*Terapon jarbua*) (66.67%) with a mean intensity of 1.0 larvae/fish ind.

4.2. Host Feeding Ecology and Age/Size

For almost all fish species, host size (in terms of length and weight) is one of the main factors affecting parasitation. Larger fish preying on infected small fish, taking a higher risk of infection and acting as accumulating hosts compared to small host fishes, which are infected only by preying on the first intermediate host, namely Euphausiacea. The prevalence of *Anisakis* infection in Belanger's croaker caught in the waters of the coast of Yogyakarta increased with increasing body size of fish (Figure 1). Most studies on various fish species reported a positive correlation between the host body size with the prevalence and mean intensity of infection (Quiazon *et al*., 2009; Setyobudi *et al*., 2011b; Mladineo *et al*., 2012; Bao *et al*., 2015; Pierce *et al*., 2017; Debendetti *et al.,* 2019; Setyobudi *et al*., 2019). In general, large fish had more time in their life to accumulate *Anisakis* compared to small fish; thus, the former has a higher infection rate than the latter (Abattuoy *et al.*, 2011). In addition, the total amount of different food items consumed and food habits, and also switches in feeding ecology while growing bigger (Munster *et al.*, 2015) affect the mean intensity of *Anisakis* infection on fishes. *Anisakis* infection occurs through predation; therefore, adult fish have a higher risk of infection (Mattiucci *et al.*, 2018). For example, cod from Greenland shows the apparent correlation between the size of fish and their food habits with parasitic abundance (Munster *et al.*, 2015).

4.3. Microhabitats

The *Anisakis* infection of Belanger's croaker at the coast of Yogyakarta was restricted to the body cavity. In some fish species with high infection rates, *Anisakis* larvae can also be isolated from internal organ, such as the liver, gonads, mesentery, or are attached to the intestinal wall (Mattiucci *et al*., 2018). The variation of infection in different microhabitats could be determined by species and age of fish, parasite species, and the environmental condition of the host after its capture (Lymbery and Cheah, 2007). Similarly, several studies reported that *Anisakis* larvae are mostly found in the abdominal cavity, such as in *Trichiurus lepturus* from Bali (Semarariana *et al*., 2012) and in some fishes in the Makassar Strait (Anshary *et al*., 2014) and Spain (Debendetti *et al*., 2019).

Previous studies indicated that *Anisakis* dominates the host's body cavity, and only a small proportion is found in the muscle (Palm *et al*., 2008, 2017). However, most *Anisakis simplex* (s.s.) larvae, which is the *Anisakis* species with the highest zoonotic potential, causing most anisakiasis cases worldwide, that infect chum salmon (*Onchoryncus keta*) were found in the muscle (98%), and only few (2%) were found in the abdominal cavity and liver (Setyobudi *et al*., 2011b). *Anisakis simplex* and *A. pegreffii* larvae are also known to undergo a post-mortem habitat shift migrating from internal organs towards the muscle tissue of the fish host (Šimat 2015, Cipriani *et al.*, 2016), but this is not known nor assumed for *A. typica*. On the basis of the relative distribution of infection between the abdominal cavity and the muscle of the hosts, some *Anisakis* species migrate to the muscle stimulated by fatty acid content gradients (Smith, 1983), or thermophilic. Larvae of *A. typica* have been found in the muscle tissue of *Auxis rochei*, but only 2.5% (1/40) (Palm *et al.*, 2008).

4.4. Morphology and DNA

Morphological identification of *Anisakis* isolated from Belanger's croaker in the coast of Yogyakarta was led to Type I, which is indicated by a long ventriculus and the existence of a mucron (Berland, 1961). Up to now, the presence of *Anisakis* Type II has not been reported in the Indian Ocean from the south coast of Java (Theisen 2019). Klimpel and Palm (2011) stated that *Anisakis* Type II is not found in Asian waters but has a zoogeography restricted to the Central Atlantic and South Africa.

Currently, molecular methods such as DNA sequencing and PCR-RFLP are widely used to identify *Anisakis* species (D'Amelio *et al*., 2000; Pontes *et al*., 2005). In this study, restrictions of PCR product using three enzymes (*Taq*I, *Hinf*I, and *Hha*I) were successfully used to identify *Anisakis* larvae. PCR-RFLP analysis produces the banding pattern corresponding to *A. typica*. PCR-RFLP can be used to identify species generally by observing the bands formed by digestion of restriction enzymes on the specific site of nucleotide sequence on a certain species. This simple and cheaper method is more appropriate when used for the identify process in a large amount of sample. However, to study the genetic variations of a species, the direct sequencing method was commonly used. For *A. typica*, the digestion of the ITS region using *Hinf*I produces two bands (620 and 350 bp), that using *Hha*I produces four bands (320, 240, 180, and 160 bp), and that using *Taq*I produces two bands (400 and 350 bp).

The *Anisakis* nucleotide sequencing of the mtDNA cox2 gene produced around 590 bp. The nucleotide sequencing showed that the *A. typica* that infects Belanger's croaker at the coast of Yogyakarta has genetic diversity among samples. *Anisakis* isolated from Belanger's croaker from Yogyakarta formed two groups which are closely related to *A. typica* from the Philippines water, the Gulf of Thailand, the Papua New Guinea, and closely related to *A. typica* from the Persian Gulf. Besides, this *A. typica* slightly different from those isolated from Egypt, Brazil, Japan, Adriatic Sea, even other isolates from Makassar Strait and Philippines waters. This finding is in line with Palm *et al.* (2017) regarding the possibility that there are varieties/sister species of *A. typica* (var. *indonesiensis*) isolated locally from Indonesia and adjacent waters. Eamsobhana *et al.* (2018) reported the *Anisakis*

larvae isolated from *P. tayenus* show the similar genetic lineage as the *A. typica* var. *indonesiensis*. Due to the closely related between *A. typica* isolated from the Gulf of Thailand and this finding, supposed that *A. typica* var. *indonesiensis* was distributed from Indian Ocean coast of Yogyakarta in the south to the Gulf of Thailand in the north. Phylogenetic analysis is appropriate in investigating the genetic composition of the *Anisakis* population and its biology (Jabbar *et al*., 2013). *Anisakis typica* has a wide distribution; it has infected several marine fish worldwide circumequatorial, and showed a unique distribution pattern (Klimpel and Palm, 2011). It is distributed from 35–40°N to 36°S in warm and tropical climates. The adult stage of *A. typica* is found in marine dolphins (Mattiucci and Nascetti, 2008; Colon-Llavina *et al*., 2009; Iniguez *et al*., 2009; Klimpel and Palm, 2011). The larval stages are found in many marine fish species and are known to have infected several fishes in Western Indonesia, Japan, Taiwan, Papua New Guinea, the Adriatic Sea in Croatia, China, Brazil, Portugal, Morocco, Mauritania, and several regions in the Mediterranean Sea (Zhu *et al.*, 2007; Chen *et al.*, 2008; Farjallah *et al*., 2008; Palm *et al*., 2008, 2017; Umehara *et al*., 2010; Borges *et al.*, 2012; Smrzlic *et al.*, 2012; Koinari *et al*., 2013; Palm et al., 2017; Theisen 2019). Infection by *A. typica* was also reported in marine mammals, i.e. dwarf sperm whales, *Kogia breviceps* from the Brazilian Atlantic coast (Iniguez *et al.*, 2011) and *Sotalia fluviatilis* from the Atlantic coast (Mattiucci *et al*., 2002).

4.5. Human Impact, Biohazard and Zoonosis

Anisakiasis, which is mostly caused by *A. simplex* and *A. pegreffii*, has been reported in various Asian and European countries (Baird *et al*., 2014; Aibinu *et al*., 2019). Anisakiasis caused by *A. typica* infection is uncommon. Reports on the presence of *A. typica* in humans suffering from anisakiasis are unknown; thus, its zoonotic effect may be ignored. However, Palm *et al.* (2008) indicated that aside from the body cavity, *A. typica* can also be found in muscles of fish hosts; therefore, these parasites might cause anisakiasis by way of consumption of raw or undercooked fish. Anisakiasis has been widely reported around the world (Lymbery & Cheah, 2007). More than 90% of anisakiasis cases originate in Asia, especially Japan, but several cases have also occurred from western countries (Audicana & Kennedy, 2008; Mattiucci *et al*., 2018). Report on human health risk due to *Anisakis* larvae infection in Indonesia is still rare. However, seropositive against excretory-secretory antigen of third-stage larvae of *Anisakis* spp. have been found from 11% of inhabitants in Sidoarjo, East Java (Uga et al., 1996).

This study indicated that various fish species in Indonesia are susceptible to *Anisakis* infection and new hosts can be recorded locally; however, still only a small number compared to the fish species inhabiting Indonesian waters is investigated. Therefore, further investigation is necessary to understand the geographical distribution, prevalence, and epidemiology of the local *Anisakis*.

5. Conclusion

Belanger's croaker at the coast of Yogyakarta was susceptible to *Anisakis* infection by a moderate prevalence (48.7%) with a mean intensity of 5.7 larvae/fish individual. All *Anisakis* larvae were found in the abdominal cavity (100%). Molecular analysis identifies those larvae as *A. typica (*var. *indonesiensis*).

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A comparative Study of Onion Purple Blotch (Caused by *Alternaria porri*) and Tomato Early Blight (Caused by *A. solani*) Diseases in Southern Ghors of Jordan

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Abstract

Two species of *Alternaria* were collected from southern Ghors of Jordan, isolated and characterized: *A. porii*, the causal agent of purple blotch of onion, and *A. solani*, the causal agent of early blight of tomato. Among planted onion cultivars, Beit Alpha was found to be highly susceptible to purple blotch, and Tebal was highly resistant under field conditions. Morphologically, there were distinct differences in conidia and mycelial growth rates between *A. porri* and *A. solani. Alternaria porii* was able to infect tomato and cause severe symptoms on its leaves; however, *A. solani* was not able to infect onion *in vitro.* Copper sulfate was the most effective salt in reducing the growth of both species *in vitro* and completely inhibited their fungal growth.

Keywords: Disease management, fungal diseases, plant pathogens, vegetable crops.

1. Introduction

Alternaria is a fungal genus that belongs to class Ascomycetes (formerly, classified in domain Eukaryota, kingdom Fungi, phylum Deuteromycota, class Hyphomycetes, order Hyphales, series *Porosporae* (Ellis & Gibson, 1975) and includes 299 species (saprophytic and pathogenic species). They are environmentally ubiquitous and are a part of common fungal flora that act as natural agents of decay and decomposition. About 20% of agricultural spoilage and decay are caused by *Alternaria* species. Crop losses caused by *Alternaria* species may reach up to 80% of the yield (Kirk *et al.,* 2008; Nowicki *et al.,* 2012). Variability is a well-known phenomenon in genus *Alternaria* and was noticed as changes in spore shape and size, mycelial growth, sporulation and pathogenicity (Mohsin *et al.,* 2016). Based on phylogenetic and morphological studies, *Alternaria* is currently divided into 26 sections (Woudenberg *et al.,* 2015). *Alternaria* sect. *porri* is the largest section containing most *Alternaria* species that have medium to large conidia and long beaks, some of which are important plant pathogens. The *porri* clade includes *A. porri* (Ellis) Cif. which causes purple blotch disease of onion as well as *A. solani* which causes early blight disease of tomato (Simmons, 1995).

Tomato (*Solanum lycopersicum* L.) is an economic solanaceous vegetable crop of high importance worldwide. Tomato is grown in an area of more than 5 million hectares around the world (WPTC, 2018) and of about 24500 hectares in Jordan with a local production of about 1.7 million tons (Anonymous, 2016). Onion (*Allium cepa* L.) is an important bulbous vegetable crop of a high global importance grown in an area of 3.7 million hectares (FAO, 2010). In Jordan, onion occupies an area of 5700 hectares that produce about 12200 tones of yield (Anonymous, 2016).

Early blight (target leaf spot) disease of tomato caused by *A. solani* is one of the world's most destructive diseases which affect tomato crops. The causal organism is air-borne and soil inhabiting. It is responsible for early blight, collar rot and fruit rot of tomato (Datar and Mayee, 1981). Symptoms of the disease usually appear on leaves, stems, petioles, twigs and fruits under favorable conditions resulting in defoliation, drying off of twigs and premature fruit drop; thus, the disease causes 50-86% loss in fruit yield (Mathur and Shekhawat, 1986).

Purple blotch of onion is a major disease throughout the world including Jordan, which is caused by *A. porri* (Islam *et al.,* 2001). This disease can cause 30-50% yield reduction (Pascua *et al.,* 1997). It causes extensive damage to bulb- as well as seed-crops. It is also a major limiting factor in the cultivation of onion (Savitha *et al.*, 2014; Priya *et al.,* 2015, Ramesh *et al.,* 2017).

Conidia, chlamydospores mycelia of *A. solani* and *A. porri* survive on plant debris and in soil. Both species have typical dry-dispersed conidia that are produced away from the host surface on aerial conidiophores (Fitt *et al.,* 1989; Everts and Lacy, 1996). Atmospheric temperature, humidity, wind speed and conidial spore concentration are the factors that are closely correlated with *A. solani* and *A.*

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porri disease occurrence. Wind, rain and insects are the primary source of disseminating inoculum of the pathogen (Rotem, 1994).

Presently, the two diseases are mainly managed by chemical fungicides, although the international trend is shifting towards more environmentally safe measures of plant disease control (Sallam, 2011; Savitha *et al.,* 2014).

The objectives of this study are to isolate and characterize the species of *Alternaria*, which cause diseases of onion and tomato grown in southern Ghors' open fields and to investigate the effects of different treatments on the suppression of the characterized pathogenic *Alternaria* species *in vitro*.

2. Materials and Methods

2.1. Field work

Several field trips were made to southern Ghors of Jordan, which is a common agricultural area under drip irrigation planted with tomato and onion crops during the pre-harvest stage of 2017/2018 fall. Visual disease observations of tomato and onion were carried out, particularly for diseases caused by *Alternaria* under open field conditions. Many diseased-plant samples were collected for laboratory pathogen isolation and identification. Disease incidence was assessed as the proportion % of diseased plants. Disease severity was scored by following 0-5 scale (Sharma, 1986) and further as a per cent disease index that was worked out by using a formula proposed by Wheeler (1969). Loss in crop yield was visually estimated in the field as the percentage of reduction in bulb size due to purple blotch disease.

2.2. Laboratory work

2.2.1. Pathogen isolation and characterization

Pathogen isolation was done by taking some superficial fungal growths of *A. solani* and *A. porri* from leaf lesions showing the typical symptoms of early blight of tomato and purple blotch of onion, respectively. They were cultured on potato dextrose agar (PDA) medium at 25°C in darkness for two weeks. The culture was then visually examined after slide preparation under a stereoscopic microscope at 100 and 400X for conidial morphology and characterization.

2.2.2. Pathogenicity test

Pathogenicity of the two *Alternaria* species was tested on tomato and onion leaves *in vitro*. A 5 cm-in-diameter piece of tomato (GS12, a susceptible cultivar) compound leaf or mature onion (Beit Alpha, a susceptible cultivar) leaf was rinsed in sterile distilled water, plated on Petri-dish and sprayed with a conidial suspension (at 10^6 conidia per ml) of each pathogen. Three plates were assigned for each treatment as replicates and three plates were sprayed with distilled water as non-treated control. All inoculated plates were incubated at 25°C and 16/8 light/dark inside a plant growth chamber for one week. Disease-symptoms development was visually monitored and photographed at the end of the incubation period.

2.2.3. In vitro fungicide bioassay

To investigate the effects of different chemical treatments on the suppression of the two pathogenic species *in vitro*, homogenized solutions of copper sulfate (at 2.5 g/l), sulfur (80% of a commercial product (c.p.) at 7 g/l), carbendazim (50% of c.p. at 2.5 g/l) and thiophenate methyl (70% c.p. at 1 g/l) were prepared under laboratory conditions. A 0.5 cm-in-diameter disc was taken from a pure culture of *A. porri* or *A. solani* and placed on the center of a freshly prepared PDA plate after treating it uniformly with 0.5 ml of each of the previous solutions. Five treated and cultured plates were assigned as replicates for each treatment and five untreated cultured plates were kept as a control. All plates were incubated at 25°C in darkness for one week. After one week, the diameter of the fungal growth on the plate of each species per treatment was measured using a 20 cm-in-length ruler and compared with that of the untreated control.

2.3. Statistical Analysis

Data was analyzed statistically using regression models where general linear model (GLM) procedure was implemented (SPSS software version 11.5; SPSS Inc., Chicago, USA). Least significance difference (LSD) test and t-test were applied for mean separation at the 0.05 probability level (Steel *et al.*, 1997).

3. Results

3.1. Field work

All planted tomato cultivars were found highly susceptible to early blight caused by *A. solani* as indicated by high disease incidence (about 85%) and severity (about 62%). No tomato cultivars resistant or tolerant were noticed during the trips in southern Ghors. Onion cultivar Tebal was found highly resistant to purple blotch (the disease incidence and severity were less than 15 %) compared to another cultivar (Beit Alpha) that was highly susceptible with a disease incidence and severity higher than 85% (Table 1). Purple blotch of onion was a highly destructive disease that had caused high economic losses especially in the susceptible cultivar Beit Alpha with approximately 39.3 % compared to 4.2 % bulb reduction in the resistant one, Tebal.

Table 1. Disease incidence and severity of purple blotch disease on two onion cultivars at fully mature bulb stage under field conditions of southern Ghors of Jordan:

Cultivar	Disease incidence %	Disease severity %
Beit Alpha	87.3 [1] a[2]	91.7 a
Tibal	12.8 b	13.6 b

[1] Average of 10 plants/ treatment

[2] Means within columns followed by the same letters are not significantly different at 0.05 probability level using t-test.

3.2. Laboratory work

3.2.1. Pathogen isolation and characterization

Morphologically, there were distinct differences in conidia morphology and mycelial growth rates between *A. porri* and *A. solani* (Figure 1). The conidia of *A. solani* are relatively larger in size and their cells are more swollen than that of *A. porri*. Furthermore, the growth rate of *A. solani* was faster than *A. porri*. The mycelium of *A. solani* was able to cover the whole plate within a week (see the control plates, Figure 3).

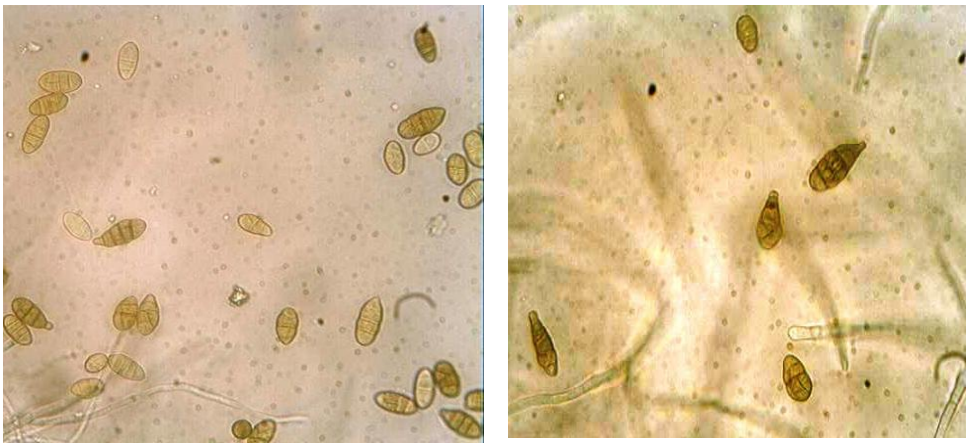


Figure 1. Conidia of *Alternaria porii* (left) and conidia of *A. solani* (right) under 400X total magnification.

3.2.2. Pathogenicity test

Alternaria porii was able to infect tomato and cause severe symptoms on its leaves, but *A. solani* was not able to infect onion *in vitro* (Figure 2). The pathogenicity of *A. porri* on tomato was confirmed by the positive re-isolation of the pathogen from symptomatic tomato leaves showing wide light brown lesions surrounded by wide yellow halo at the end of incubation period (Figure 2).

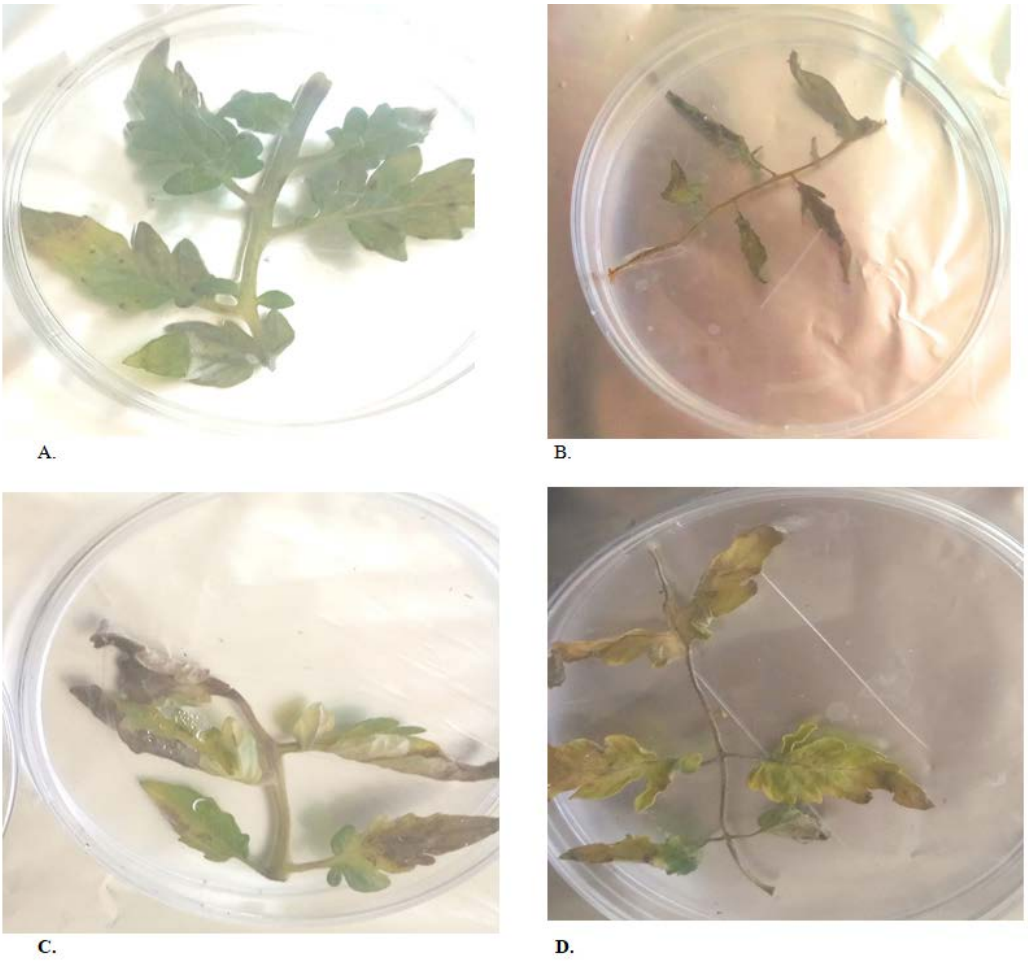


Figure 2 Symptoms resulted from artificial inoculation and infection of tomato with *Alternaria porii* (in A: after one week and in B: after two weeks) or with *A. solani* (in A: after one week and in B: after two weeks).

3.2.3. In vitro fungicide bioassay

Copper sulfate was the most effective treatment. It was more effective than sulfur and even than the two systemic fungicides; carbendazim and theophenate methyl in reducing the growth of *A. porri* and *A. solani* in the bioassay test. Copper sulfate treatment completely inhibited the fungal growth of both *Alternaria* species *in vitro* (Figures 3 & 4).

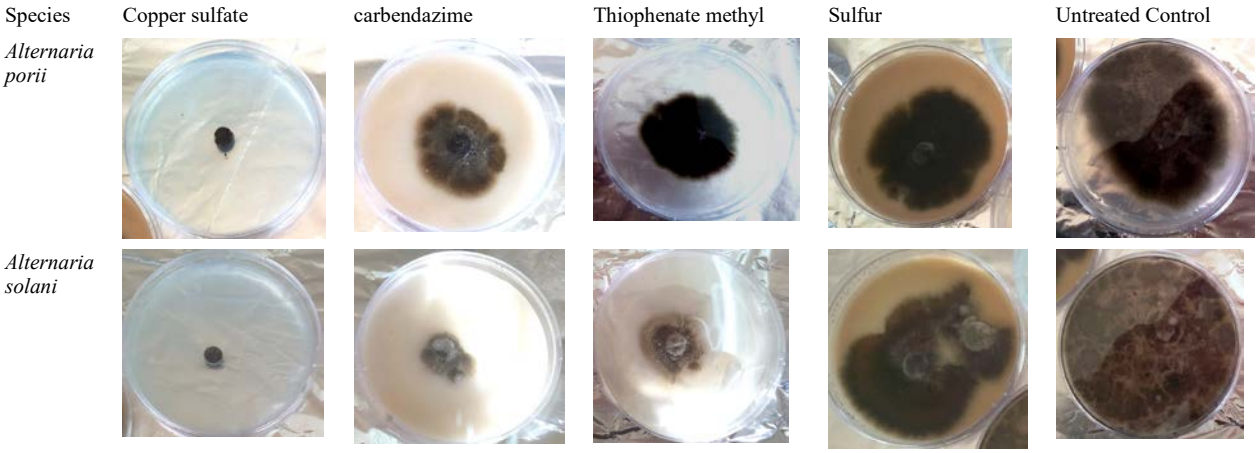
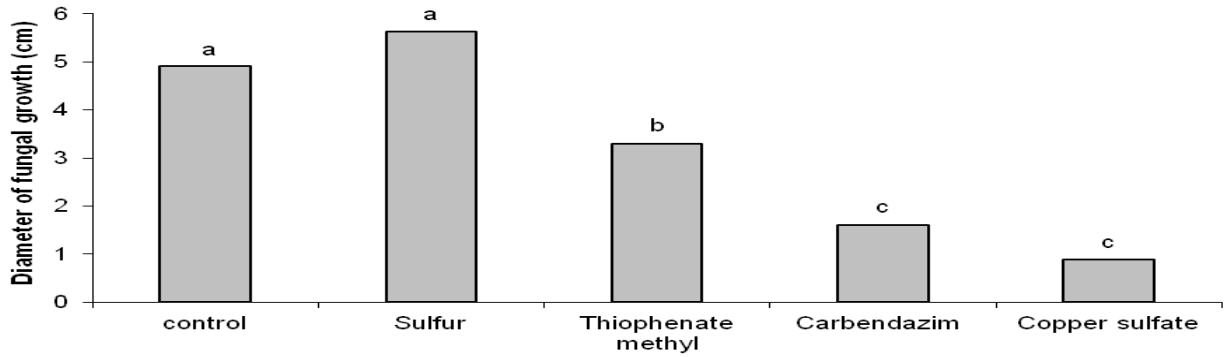


Figure 3: Fungal growth of *Alternaria porii* and *A. solani* on PDA treated with different chemical treatments.

A. Alternaria solani:



B. Alternaria porii:

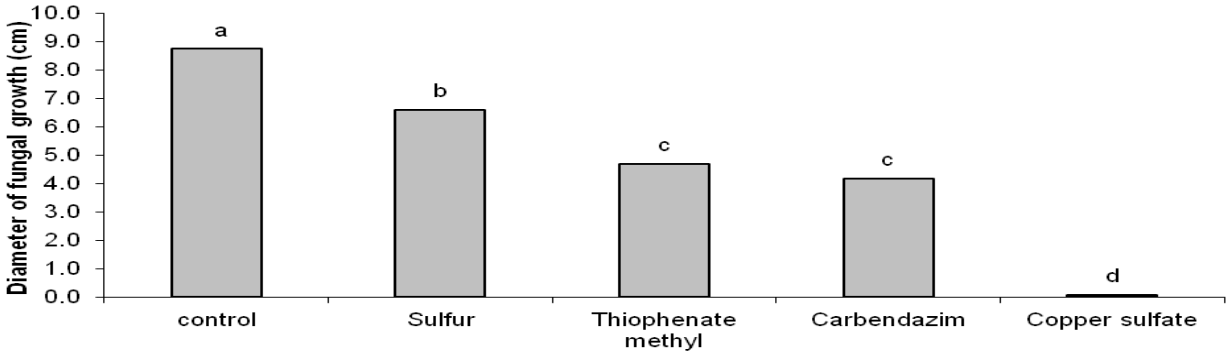


Figure 4: Fungal growth (in cm) of *Alternaria solani* and *A. porii* on PDA treated with different treatments.

4. Discussion

The two diseases caused by *A. solani* and *A. porii*, common in southern Ghors of Jordan, resulted in considerable plant-yield losses as indicated from our field observations. The influence of purple blotch disease was more severe in the susceptible onion cv. Beit Alpha than cv. Tebal which appeared to be resistant. Therefore, the use of resistant cultivars of onion could be a useful, easy-to-use and effective tool to reduce crop losses due to the disease. The use of resistant cultivars of tomato could be useful also in tomato since no cultivars resistant or tolerant to early blight disease were noticed during the trips in southern Ghors. It might be due to the loss of high genetic variability. Low genetic resistance to pest and diseases in commercial tomato cultivars induces farmers, under conventional crop systems, to use high levels of pesticides that do not meet consumer requirements (Modolon *et al.,* 2012). Epidemics do not generally occur until late in the season, when the plants are most susceptible. However, disease-progress curves differ depending on location and prevailing weather conditions.

Early blight can be controlled by effective use of cultural practices such as a 3-5 year crop rotation with non-host crops or resistant cultivars, site selection, sanitation of fields, providing proper plant nutrition, avoiding water stress and planting disease-free seed (Madden *et al.,* 1978). Generally, the best crops for rotation are forage crops and grains, including maize. Successive or consecutive cropping of tomato or potato in one field promotes an earlier appearance of early blight (Shtienberg and Fry, 1990).

As it is revealed by the morphological observations, the two *Alternaria* species (*A. solani* and *porii*) have

variations in the shape and size of conidia. In addition, there are differences in the growth rate between them on PDA medium. These results agree with the previous studies (Ellis & Gibson, 1975; Pryor and Gilbertson, 2000; Hussein, 2019).

Alternaria porii was able to infect tomato and causes severe symptoms on its leaves but *A. solani* was not able to infect onion *in vitro*. The pathogenicity of *A. porri* on tomato was confirmed by the positive re-isolation from symptomatic tomato leaves showing wide light brown lesions surrounded by wide yellow halo. In a study using ITS and mitochondrial SSU sequences of *Alternaria* species,) showed a distinct number of species-clades. Although the porri clade includes both *A. porri* and *A. solani* as related species, there are differences in their pathogenicity to different hosts that could be due to phylogenetic and enzymatic variability (Pryor and Gilbertson, 2000; Hussein, 2019).

Our study revealed that both *Alternaria* species (*A. porri* and *A. solani*) were destructive plant pathogens. This result has an agreement with the previous studies (Pelletier, 1988; Shuman, 1995). *Alternaria solani* is a polycyclic pathogen as many cycles of infection are possible during a season (Shuman, 1995). Primary infections on new plantings of potatoes or tomatoes are caused by over-wintering inoculum (Pscheidt, 1985). The pathogen over-winters as mycelium or conidia in plant debris, soil, infected tubers and fruits or other host plants (Pelletier, 1988; Shuman, 1995; Al-Ameiri *et al.,* 2015).

Among tested salts and fungicides, copper containing fungicides (e.g. copper sulfate) could be effective in protecting plants from both diseases. A number of foliar fungicides can be used to manage early blight of tomato as they were effective. Copper-oxychloride, mancozeb and chlorothalonil are the most frequently used protectant fungicides for early blight management but they provide insufficient control under high disease pressure (Holm *et al.,* 2003; Pasche and Gudmestad, 2008).

5. Conclusion

Both *Alternaria* species (*A. solani* and *A. porii*) were found naturally occurring in southern Ghors of Jordan and causing purple blotch of onion and early blight of tomato that resulted in considerable yield losses. Onion cv. Tebal was found to be resistant to purple blotch under field conditions. *Alternaria porii* was able to infect both crops *in vitro*. Copper sulfate was effective in the suppression of fungal growth of both *Alternaria* species *in vitro*.

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Suppression of *Aspergillus* and *Penicillium* Species by Organic Extracts of Common Soft Corals and Sponge Species Habited in Hurghada, Red Sea, Egypt

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Abstract

The ethanolic extracts of eight soft corals and five sponges were screened for antifungal activity (AU) against pathogenic fungi infected corals. The data revealed that the AUs, recorded by different crude extracts (ethanol and ethyl acetate) of soft corals, ranged between 0.0 and 49.0, which was achieved by the extract of *S. gracile* against *A. fumigatus*. In addition, the values of AUs, recorded by different crude extracts (ethanol and ethyl acetate) of sponge species, revealed that the AU ranged between 0.0 and 36.0, which was achieved by extract of *Suberea mollis Row* against *P. auratiogriseum.* Cleary, our findings proved that both ethanolic and ethyl acetate extracts of soft corals (*S. polydactyla, S. gracile, S. glaucum, S. trocheliophorum, S. ehrenbergi,* and *X. macrospiculata*) were the most effective extracts that exhibited the most potent AUs. Moreover, the most effective extracts were tested to determine the MICs against the most affected fungi and the lowest effective MIC was 10 mg/mL against several pathogenic fungi. On the other hand, the GC/MS profiles of the most effective extracts were: fatty acids and their derivatives, terpenoid (Nootkaton-11,12-epoxide, caryophyllene, geranyl-a-terpinene, etc.), steroid (corticosterone, cis-calamenene, etc.), and others. An additional trail proved the efficacy of palmitic acid (as a potent example of bioactive constituents found in crude extracts) when was compared to Treflucan.

Keywords: Antifungal Activity, Organic Extract, Soft Corals, Sponge, Red Sea.

1. Introduction

Marine invertebrates have been considered as potent resources for bioactive metabolites that are new in structure and biologically active. Potential bioactivities of numerous compounds extracted from marine species include: anti-fungal, antibacterial, antiviral, antimalarial, anti-cancer, antihelmintic, anti-inflammatory, anticoagulant, and other bioactivities (Somnath and Ghosh, 2010; Datta *et al.,* 2015). Soft corals, besides sponges, are among common marine invertebrates which have been concerned for new bioactive substances (Ibrahim *et al.*, 2012; Liu *et al*., 2019).

In particular, soft corals are known to produce a wide array of secondary metabolites, particularly diterpenoids and steroids, and often characterized by uncommon structural features and potent bioactivities (Liu *et al*., 2019). The remarkable abundance and diversity of bioactive small molecule which have been isolated from soft corals have made these organisms an important source of new drug candidates for human diseases, particularly for their anti-inflammatory activity (Putra and Murniasih, 2016). For instance, Kelman *et al*. (1998) studied different developmental and reproductive features of the Red Sea soft coral; *Parerythropodium fulvum,* which showed antimicrobial activity towards many co-occurring and potentially pathogenic marine bacteria. Cheng *et al*. (2009) obtained antimicrobial activities from the soft coral; *Nephthea erecta* and *Nephthea chabroli*. Furthermore, Ibrahim *et al.* (2012) examined the extracts of ten soft corals from Egyptian Red Sea, which exhibited clear antibacterial activities against tested pathogens.

In addition, sponges are considered promising resources to supply future treatments for sever diseases such as cancer, a number of viral diseases, malaria and inflammation (Ibrahim *et al.,* 2018). Sponges produce a different kind of chemical substances with numerous carbon skeletons (such as fatty acids, terpenoids, alkaloids, polyketides, polyacetylenes, sterols, peptides, etc.), which have been found to be the main component interfering with human pathogenesis at different sites. The fact that different diseases have the capability to fight at different sites inside the body can increase the chances to produce targeted medicines (Anjum *et al*., 2016). Many of such compounds possess antibacterial, antiviral, antifungal, antimalarial, antitumor, immunosuppressive, and cardiovascular activity (Anjum *et al*., 2016), besides antifouling properties (Qian, *et al.,* 2006).

Thus, the current work investigated the suppression of *Aspergillus* and *Penicillium* growth *in vitro* by marine bioactive products extracted from soft corals and sponges

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species in Hurghada, Red Sea, Egypt. In addition, this study extended to determine the general nature of the most potent compounds via gas chromatography/mass spectrometry (GC/MS) technique.

2. Materials and methods

2.1. The study area

Marine living samples were collected away from 5 km a way of Hurghada city centre, Egyptian Red Sea. This area is adjacent to the National Institute of Oceanography and Fisheries, Red Sea branch, at latitudes of 27˚ 17' 13" N and longitudes of 33˚ 46' 43" E. It extends to about 150 m seawards and ends with a lagoon of 5 m depth. This area forms a lagoon, which has a sandy bottom and is covered with algal and sea grass mats. The reef following it is ribbon-like and composed of many hard and soft coral species.

2.2. Fungal culture and reference fungi

Potato dextrose agar (PDA) medium was applied to isolate and culture fungi. It was also used in the suppression trails. Pathogenic fungi isolated from infected hard corals were identified by the same authors and then used as reference pathogenic strains. However, they were: *Aspergillus niger, A. flavus, A. terreus, A. parasiticus, A. fumigatus, Penicillium chrysogemnum, P. oxalicum*, *P. crustosum, P. aurantiogriseum*, and *P. echinulatum* (El-Morsy *et al*., 2017).

2.3. Sampling and identification marine organisms

SCUBA and snorkeling diving were followed to collect eight soft coral and five sponge samples. Soft corals species were identified according to Reinicke (1995) and Fabricious and Aldersdale (2001), while sponges' species were identified according to Collin *et al.* (2005)., including; morphological characteristics such as the color, size, shape, and form of the sampled specimens were examined and compared. Underwater images are especially important because soft corals emit different colors in the water. Moreover, certain organs in the specimen were observed under light microscope (Olympus CX41 with 100× and 400× magnification).

2.4. Preparation of different organic extracts

With 200 mL of ethanol and ethyl acetate solvent, 80 g of each marine sample (sponges and soft coral) were macerated well. They were filtered by filter paper after soaking for two weeks. In order to obtain crude extracts, solvents were evaporated using a rotary evaporator **(**Ballantine, 1987). Following the Backus and Green Process, bioactive substances were extracted from the sponge with some modifications. A weight of 5 g of the sponge was placed in 100 mL methanol. The quantity of ethanol and ethyl acetate used was 20 times as much as the sponge weight (5%, w/v). After the solution filtration, the residue was dried in the air in the room and then stored in a 4°C refrigerator until the next level (Hutagalung *et al.,* 2014).

2.5. Antifungal activity of different extracts against reference fungi

The suppression of different extracts from selected sponges and soft corals against the isolated fungi was assessed by the well-cut diffusion method. Three hundred milliliters of PDA medium were prepared and then inoculated with 1.5 mL of fungal spore suspension (10^6 spores/mL). Fifty millimetres of PDA were poured into all plates. After solidifying, 5 mm-wells were punched out and 100 µL of extracts were moved into each well. At 28ºC, all plates were incubated for 5 days. A good result was shown by measuring the radius of inhibition zone (Y) around the each well (X) linearly in mm after the incubation period. The activity was expressed as absolute unit (AU) was calculated by dividing Y^2 per X^2 (El-Masry *et al*., 2002).

2.6. Minimal inhibitory concentrations (MICs)

The MICs were also determined by well-cut diffusion method through preparing five concentrations in mg/mL (10, 30, 50, 100, and 200) for each extract against tested fungi (*A. niger, A. terreus, A. fumigatus, P. chrysogenum, P. oxalicum,* and *P. aurantiogriseum*). All extracts were previously prepared as stock solutions (500 mg/mL) and then were sterilized by 0.45 µL filter and stored at 4°C. After preparing and autoclaving PDA medium, the tested fungi were amended in ratio of 5 mL spore suspension (10^6 spores/mL) per a litre of medium. The tested extracts were added in the five concentrations as mentioned and then all plates were incubated for 5 days at 28°C. It should be taken in consideration that the MIC of certain extract is determined as "the lowest concentration of extract, which inhibits visible growth of the microbe around the well or disc" (Devi *et al*., 2013).

2.7. Composition analysis of potent extracts using GC/MS spectroscopy

The crudes extracted from collected soft coral and sponge species, either in ethanol and ethyl acetate, were prepared for GC/MS analysis. Extracts prepared as mentioned before were concentrated until complete dryness and finally re-suspend in appropriate volume of solvent. However, the filtrate was subjected to GC/MS analysis (Perkin Elmer, Waltham, MA, USA) according to Muller *et al*. (2002) and Thakur and Pandey (2016) procedures.

2.8. Effect of common constituents in extracts and antifungal drug (Treflucan)

From the GC/MS profile of ethanolic and ethyl acetate extracts, the presence of many potent compounds [such as; 6-octen-1-ol, 3,7-dimethyl-, (R)- (citronellol); 5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z); pentadecanoic acid, 14-methyl-, methyl ester (palmitic acid); Trans-13-octadecenoic acid, methyl ester (Stearic acid); tridecanoic acid, methyl ester] was revealed as the common constituents in both extracts. The commercial palmitic acid was selected to be examined against the most affected fungi during the screening tests in comparison to commercial antifungal drug (Treflucan). However, the fungal discs of strains *A. niger* and *P. auratiogriseum* were inoculated individually on the top of the sterilized PDA plates according to Amer and Ibrahim (2019). The punched wells were filled with different concentrations of palmitic acid and Treflucan. Later, all plates were incubated at 28°C for 5 days. The results were obtained by measuring the diameter of inhibition zone around each well in millimeter.

2.9. Statistical treatment

Data were expressed as mean of three readings ± SD and was treated with a ANOVA test using SPSS software version. A P-value of < 0.05 is considered as significant value.

3. Results and discussion

3.1. Identification of marine organisms

Eight soft coral species collected from Red Sea were identified as: *Nephthya pacifica, Sarcophyton ehrenbergi, S. glaucum, S. trocheliophorum, S. gracile, Sinularia polydactyla, Xenia macrospiculata*, and *Dendronephthya klunzingeri*. Also, five selected sponge species collected from Red Sea were identified as: *Hyrtios erectus*, *Calliospongia* sp., *Amphimedon* sp., *Suberea mollis*, and *Ircinia* sp. However, macrographs of these species are shown in Figure 1.

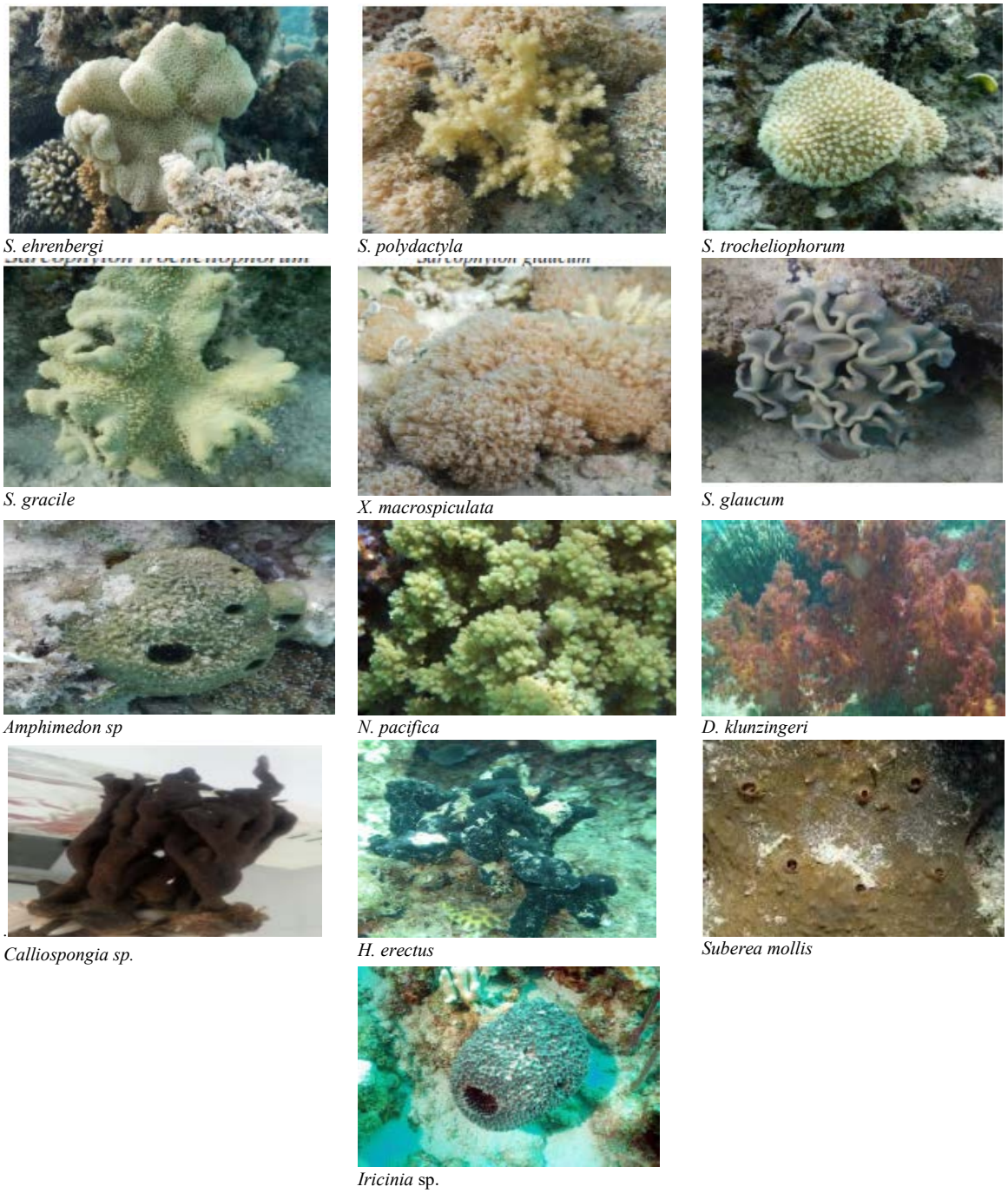


Figure 1. Selected soft coral and sponge species used in screening the antifungal activity, collected from Egyptian Red Sea, at Hurghada city.

Mycopathogens of aquatic animals have become the focus of considerable attention because of the high occurrences of fungal diseases in wild populations and aquaculture. Most marine fungal infections, once established in an individual, are often lethal and difficult to treat. This suggests that these fungi will continue to be troublesome pathogens of marine organisms (Noga, 1990). In comparison, marine species, such as sponges, soft corals, sea hares, bryozoans, sea slugs, and marine microorganisms, etc., are rich sources of novel bioactive metabolites (Blunt *et al*., 2005). Recently, many studies have confirmed the antibacterial, antifungal and antiviral activities of bioactive compounds extracted from marine organisms (Somnath and Ghosh, 2010; Ibrahim *et al*., 2018; 2020a; 2020b).

3.2. Screening of antifungal activity of coral extracts

Preliminarily, the present study investigated the inhibition of the fungal growth *in vitro* through applying selected marine bioactive extracts came from common Red Sea soft corals and sponges, Egypt.

In general, ethanolic extracts of eight soft coral and five sponge species were then screened for antifungal activity expressed as AU using well-cut diffusion technique. Result shown in Table 1 presents the AU recorded by different extracts (ethanol and ethyl acetate) of soft coral species against tested fungi. However, data revealed that the AU ranged between 0.0 and 49.0, which was achieved by the extract of *S. gracile* against *A. fumigatus*. This AU value was followed by 42.3 for extracts of both *S. glaucum* and *S. trocheliophorum* against *P. auratiogriseum* and *A. fumigatus*, respectively. Relatively, there were many other high records. The lowest value, rather than negative records, was 1.4 against *P. echinulatum*, which was given by extract of *X. macrospiculata*.

In addition, result shown in Table 2 presents the AU recorded by different extracts (ethanol and ethyl acetate) of sponge species against tested fungi. However, data revealed that the AU ranged between 0.0 and 36.0, which was achieved by extract of *Suberea mollis* against *P. auratiogriseum.* This value was followed by *Amphimedon* sp. extract against both *P. oxalicum* (AU = 31.6) and *P. crustosum* (AU = 30.0). However, there were many other relatively high records. Also, the lowest value, rather than negative records, was 2.0 against *P. echinulatum* which was occurred by extract of *Calliospongia sp.*

Table 1: Screening of antifungal activity for soft coral species extracts by well-cut diffusion technique (AU) against both *Aspergillus* and *Penicillium* species.

1, *A. flavus*; 2, *A. niger*; 3, *A. fumigatus*, 4, *A. parasiticus*; 5, *A. terreus*; 6, *P. auratiogriseum*; 7, *P. chrysogenum*; 8, *P. oxalicum*; 9, *P. echinulatum*; 10, *P. crustosum*.

Species/extract	Antifungal activity (AU)									
	1	2	3	4	5	6	7	8	9	10
Ethanol crude extract:										
D. klunzingeri	0.0±0.0	9.6±0.17	4.4±0.06	3.6±0.10	0.0±0.0	0.0±0.0	4.4±0.0	0.0±0.0	1.2±0.06	6.8±0.06
N. molle	6.3±0.06	9.0±0.10	23.0±0.17	8.4±0.15	1.4±0.0	0.0±0.0	4.8±0.12	0.0±0.0	1.4±0.12	4.0±0.21
S. ehrenbergi	7.8±0.06	7.3±0.15	13.7±0.20	6.3±0.25	12.3±0.21	4.4±0.20	4.0±0.0	4.0±0.15	9.0±0.26	4.0±0.21
S. glaucum	6.8±0.21	4.8±0.15	5.8±0.10	4.8±0.06	0.0±0.0	0.0±0.0	12.30.15	12.3±0.15	13.7±0.25	12.3±0.15
S. trocheliophorum	4.0±0.12	5.8±0.21	5.8±0.12	0.0±0.0	4.0±0.20	0.0±0.0	4.0±0.12	12.3±0.15	16.0±0.12	7.80.10
S. gracile	5.3±0.15	4.0±0.15	6.8±0.26	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	9.0±0.25	0.8±0.0	0.0±0.0
S. polydactyla	17.6±0.31	17.6±0.12	23.0±0.21	10.9±0.17	21.2±0.15	24.0±0.15	16.0±0.12	25.0±0.15	16.0±0.21	7.8±0.15
X. macrospiculata	4.8±0.06	5.3±0.21	10.9±0.12	8.4±0.21	5.3±0.12	4.8±0.12	9.0±0.06	8.4±0.06	12.3±0.12	12.3±0.06
Ethyl acetate crude extract:										
D. klunzingeri	0.0±0.0	6.8±0.06	4.0±0.18	0.0±0.0	0.0±0.0	0.0±0.0	2.6±0.06	0.0±0.0	5.3±0.12	4.0±0.15
N. molle	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
S. ehrenbergi	5.8±0.10	10.9±0.15	5.7±0.23	9.0±0.10	20.3±0.17	2.6±±0.0	16.0±0.21	5.3±0.12	10.9±0.15	4.0±0.12
S. glaucum	2.6±0.0	22.1±0.23	36.0±0.29	16.0±0.15	20.3±0.20	3.2±±0.06	36.0±0.30	4.0±0.20	20.3±0.25	6.3±0.21
S. trocheliophorum	2.6±0.0	4.0±0.0	42.3±0.87	3.6±0.15	4.4±0.0	0.0±0.0	0.0±0.0	4.0±0.15	0.0±0.0	6.3±0.15
S. gracile	4.8±0.15	6.5±0.20	49.0±1.04	7.3±0.10	25.0±0.36	0.0±0.0	30.3±1.01	4.0±0.20	32.5±1.51	0.0±0.0
S. polydactyla	5.8±0.10	9.0±0.31	10.9±0.89	4.4±0.06	6.3±0.26	4.0±0.15	12.3±0.15	5.3±0.20	16.0±0.36	4.0±0.0
X. macrospiculata	4.0±0.15	9.0±0.31	10.2±0.12	6.3±0.42	7.3±0.12	3.2±±0.0	4.8±0.26	0.0±0.0	1.4±0.0	4.0±0.10

Actually, soft corals have been investigated by scientists globally for their diversity of chemical constituents and biological activities (Liang and Fang, 2006). Bowden *et al*. (1984) discovered that soft coral;

Lobophytum crasscopiculatum yielded cembranolides compound, which have antimicrobial activities. Kelman *et al*. (1998) studied the Red Sea soft coral; *Parerythropodium fulvum,* which exhibited antimicrobial activity against several co-occurring and potentially pathogenic marine bacteria. Geffen and Rosenberg (2005) showed antibacterial from the coral; *Pocillopora damicornis*. Cheng *et al*. (2009) obtained antimicrobial activities from *N. erecta* and *N. chabroli*. Ibarhim *et al.* (2012) previously investigated number of these corals from Egyptian Red Sea (particularly; *S. acutum, S. spongosium, S. gracile, S. glaucum, Sinularia gardineiri, S. leptoclados, Lopophytum pauciliforum, Dendronephthea sp., N. pacifica,* and *X. macrospiculata*). However, their extracts showed obvious antibacterial activities against some common pathogens.

Table 2: Screening of antifungal activity for sponge species extracts by well-cut diffusion technique against both *Aspergillus* and *Penicillium* species.

Species/extract	Antifungal activity (AU)									
	1	2	3	4	5	6	7	8	9	10
Ethanol crude extract:										
H. erectus	4.9±0.10	10.9±0.15	16.0±0.55	6.3±0.12	9.0±0.21	9.0±0.35	0.0±0.0	0.0±0.0	2.2±0.12	7.3±0.17
Calliospongia	2.6±0.12	23.0±0.38	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	2.3±0.15	0.0±0.0
Amphimedon	5.3±0.25	18.5±0.90	25.0±0.75	5.7±0.06	16.0±0.44	16.0±0.55	12.9±0.20	20.3±0.75	13.7±0.30	23.0±0.82
Suberea mollis	0.0±0.0	0.0±0.0	4.0±0.06	0.0±0.0	12.3±0.35	16.0±0.30	0.0±0.0	4.0±0.15	0.0±0.0	4.0±0.15
Iricinia sp.	0.0±0.0	4.0±0.21	16.0±0.44	4.0±0.10	0.0±0.0	9.0±0.31	0.0±0.0	5.7±0.36	9.0±0.31	9.0±0.45
Ethyl acetate crude extract:										
H. erectus	3.6±0.15	23.0±0.55	13.7±0.45	10.9±0.45	9.0±0.35	13.7±0.36	16.0±0.56	16.0±0.10	16.0±0.42	16.0±0.31
Calliospongia	0.0±0.0	17.6±0.31	16.0±0.25	5.8±0.31	0.0±0.0	13.7±0.40	15.2±0.21	0.0±0.0	2.0±0.21	0.0±0.0
Amphimedon	0.0±0.0	9.0±0.50	20.2±0.51	16.0±0.65	13.0±0.31	28.1±0.56	13.7±0.31	31.6±0.20	16.0±0.20	30.0±0.56
Suberea mollis	0.0±0.0	0.0±0.0	25.0±0.46	4.0±0.10	7.8±0.32	36.0±0.50	0.0±0.0	0.0±0.0	2.3±0.06	9.0±0.46
Iricinia sp.	0.0±0.0	16.0±0.21	7.8±0.20	12.3±0.26	0.0±0.0	9.0±0.40	0.0±0.0	0.0±0.0	4.0±0.30	0.0±0.0

1. *A. flavus*; 2, *A. niger*; 3, *A. fumigatus*, 4, *A. parasiticus*; 5, *A. terreus*; 6, *P. auratiogriseum*; 7, *P. chrysogenum*; 8, *P. oxalicum*; 9, *P. echinulatum*; 10, *P. crustosum*.

Additionally, sponge extracts exhibited antimicrobial activities. For instance, sponge aqueous extract was used as antifungal activity (Perdicaris *et al.,* 2013) and antibacterial against certified strains of bacteria **(**Galeano and Martínez, 2007; Ibrahim *et al*., 2018**)**. Dhinakaran and Lipton (2012) examined the antifungal activity of the organic extracts of the sponge; *Sigmadocia pumila* against various fungal strains such as: *Trichoderma viride, Fusarium spp., A. niger, Candida albicans, P. chrysogenum,* and *A. flavis*. Hence it is assumed that the sponge exhibited high antimicrobial activities. Ibrahim *et al.* (2018) also collected five marine sponges from the Levantine Basin in the vicinity of Alexandria city, Egypt (Spongia sp., *Cinachyrella* sp., *Ciocalypta penicillus*, *Axinella verrucosa*, and *Plakortis simplex*) and their results showed that the acetone extract of *Spongia* sp. had a broad spectrum followed by extracts of *C. penicillus* and *A. verrucosa*.

More recently, Ibrahim *et al.* (2020a) studied treating pathogenic bacteria (*Escherichia coli, Klebsiella pneumonia* and *Staphylococcus aureus*) isolated from human stool and urine samples by the crudes extracted from sponge (*Negombata magnifica*, *Siphonochalina siphonella*, and *H. erectus*) and soft coral (*S. glaucum* and *X. macrospiculata*). Their data were also promising in such manner. Ibrahim et al. (2020b) evaluated the antimicrobial properties of three species of sponge collected from Red Sea, Hurghada, Egypt. They were identified as; *Cinachyrella arabica*, *Ciocalypta penicillus* and *Axinella verrucosa.* Their data revealed the positive values of antifungal and antibacterial activities in extracts, especially against *A. hydrophila, S. aureus* and *P. notatum*.

Data in both Table 1 and 2 were expressed as mean of three readings ± SD. Correlations between antifungal activities and fungal species in case of ethanol crude extracts were significant; where P-value < 0.001. Also, they were significant in case of ethyl acetate crude extracts; where P-value < 0.05. In very few cases of the latter condition, they were not significant; where P-value > 0.05 (Table 3).

Table 3: Results of the ANOVA test showing significance between type of crude extract and antifungal activity (AU) presented in Table 1 and 2.

AU/Fungal species	For data in Table 1				For data in Table 2			
	Ethanol crude extract		Ethyl acetate extract		Ethanol crude extract		Ethyl acetate extract	
	F	Significance	F	Significance	F	Significance	F	Significance
AU/1	3208.538	0.000	1946.164	0.000	1100.923	0.000	1635.571	0.000
AU/2	2227.401	0.000	1.541	0.223	1335.330	0.000	1711.002	0.000
AU/3	6310.098	0.000	1.288	0.317	1435.691	0.000	1.223	0.361
AU/4	2190.637	0.000	2747.693	0.000	5235.750	0.000	451.171	0.000
AU/5	11384.127	0.000	4.172	0.009	2271.668	0.000	1547.146	0.000
AU/6	11384.127	0.000	2940.268	0.000	1066.844	0.000	1921.672	0.000
AU/7	127.686	0.000	3593.907	0.000	12870.750	0.000	2255.653	0.000
AU/8	9916.997	0.000	902.128	0.000	1470.158	0.000	60203.400	0.000
AU/9	4928.935	0.000	1237.145	0.000	2213.636	0.000	2148.664	0.000
AU/10	2846.210	0.000	1233.613	0.000	1258.791	0.000	3847.546	0.000

1. *A. flavus*; 2, *A. niger*; 3, *A. fumigatus*, 4, *A. parasiticus*; 5, *A. terreus*; 6, *P. auratiogriseum*; 7, *P. chrysogenum*; 8, *P. oxalicum*; 9, *P. echinulatum*; 10, *P. crustosum*.

3.3. MICs of the most effective extracts

Thereafter, the MICs were determined against the most affected fungal pathogens. The MICs were detected by applying five concentrations (10, 30, 50, 100, and 200 mg/mL) for each extract obtained from soft corals (*S. polydactyla, S. gracile, S. glaucum, S. trocheliophorum, S. ehrenbergi,* and *X. macrospiculata*) against the most affected fungal pathogens *(A. niger, A. terreus, A. fumigates, P. aurantiogriseum, P. chrysogenum,* and *P. oxalicum).*

Data in Table 4 illustrates the MICs of *S. polydactyla* ethanolic extracts were 10 mg/mL against all tested *Aspergillus* species. The MICs of *S. gracile* ethanolic extracts were 30 mg/mL against *A. niger* and *A. fumigatus* and 200 mg/mL against *A. terreus.* The MICs of *S. glaucum* ethanolic extracts were 50 mg/mL against both of *A. niger* and *A. fumigatus* and 100 mg/mL against *A. terreus.* The MICs of *S. trocheliophorum* ethanolic extracts were 30 mg/mL against both of *A. niger* and 50 mg/mL against *A. fumigatus* and *A. terreus.* The MICs of *S. trocheliophorum* ethanolic extracts were 30 mg/mL against both of *A. niger* and 50 mg/mL against *A. fumigatus* and *A. terreus.* The MICs of *S. ehrenbergi* ethanolic extracts 10 mg/mL against both of *A. terreus* and *A. fumigatus* and 30 mg/mL against *A. niger* The MICs of *X. macrospiculata* ethanolic extracts 30 mg/mL against all tested *Aspergillus* species.

Table 4: MICs of extracts (mg/mL) from selected soft coral against the most previously affected *Aspergillus* species [a= concentration of extract (mg/mL), while b= zone, mm].

	MICs (mg/mL)/Fungus					
	A. niger		*A. terreus*		*A. fumigatus*	
Species/extract	Ethanol	Ethyl acetate	Ethanol	Ethyl acetate	Ethanol	Ethyl acetate
S. polydactyla	10^a	30^a	10^a	30^a	10^a	30^a
	10^b	9^b	11^b	9^b	10^b	9^b
S. gracile	30^a	30^a	200^a	10^a	30^a	10^a
	5^b	5^b	11^b	13^b	9^b	11^b
S. glaucum	50^a	10^a	100^a	10^a	50^a	10^a
	10^b	10^b	9^b	10^b	12^b	10^b
S. trocheliophorum	30^a	50^a	50^a	30^a	50^a	10^a
	5^b	10^b	11^b	8^b	11^b	11^b
S. ehrenbergi	30^a	10^a	10^a	30^a	10^a	10^a
	8^b	7^b	12^b	11^b	8^b	$9^{b\,b}$
X. macrospiculata	30^a	30^a	30^a	30^a	30^a	30^a
	10^b	9^b	10^b	8^b	11^b	10^b

On the other side, the MICs of both of *S. polydactyla* and *X. macrospiculata* ethyl acetate extract were 30 mg/mL against all tested *Aspergillus* species. The MICs of both of *S. S. gracile* ethyl acetate extract were 10 mg/mL against both of *A. terreus* and *A. fumigatus* and 30 mg/mL against A. *niger.* The MICs of both of *S. S. glaucum* ethyl acetate extract were 10 mg/mL against all tested *Aspergillus* species. The MICs of both of *S. trocheliophorum* ethyal acetate extract were 50, 30 and 10 mg/mL against A. *niger, A. terreus* and *A. fumigatus,* respectively. The MICs of both of *S. ehrenbergi* ethyl acetate extract were 10 mg/mL A. *niger* and *A. fumigatus* and 30 mg/mL against *A. terreus.*

The results in Table 5 revealed that the MICs of *S. polydactyla* ethanolic extract were recorded as 10 mg/mL against both of *P. aurantiogriseum* and *P. chrysogenum* and 30 mg/mL against *P. oxalicum*. The MICs of *S. gracile* ethanolic extract were 50, 100 and 30 mg/mL against *P. aurantiogriseum, P. chrysogenum* and *P. oxalicum,* respectively. The MICs of *S. glaucum* ethanolic

extract were 10 mg/mL against *P. chrysogenum* and 30 mg/mL against both of *P. aurantiogriseum,* and *P. oxalicum*. The MICs of *S. trocheliophorum* ethanolic extract were 30 mg/mL against *P. oxalicum* and 50 mg/mL against both of *P. aurantiogriseum* and *P. chrysogenum.* The MICs of *S. ehrenbergi* ethanolic extract were 30 mg/mL against *P. aurantiogriseum* and 50 mg/mL against both of *P. chrysogenum* and *P. oxalicum.* The MICs of *X. macrospiculata* ethanolic extract were 10, 30 and 100 mg/mL against *P. aurantiogriseum, P. chrysogenum* and *P. oxalicum,* respectively.

On the other hand, the MICs of *S. polydactyla* ethyl acetate extract were recorded to be 10 mg/mL against *P. aurantiogriseum* and 30 mg/mL against both of *P. chrysogenum* and *P. oxalicum.* The MICs of both of *S. gracile* and *S. glaucum* ethyl acetate extract was recorded to be 10 mg/mL against *P. aurantiogriseum* and 50 mg/mL against both of *P. chrysogenum* and *P. oxalicum.* The MICs of both of *S. trocheliophorum* ethyl acetate extract was recorded to be 10, 100 and 50 mg/mL against *P. aurantiogriseum, P. chrysogenum* and *P. oxalicum,* respectively. The MICs of both of *S. ehrenbergi* ethyl acetate extract was recorded to be 30 mg/mL against *P. aurantiogriseum* and 50 mg/mL against both of *P. chrysogenum* and *P. oxalicum,* The MICs of both of *S. ehrenbergi* ethyl acetate extract was recorded to be 30 mg/mL against all tested *Penicillium* species.

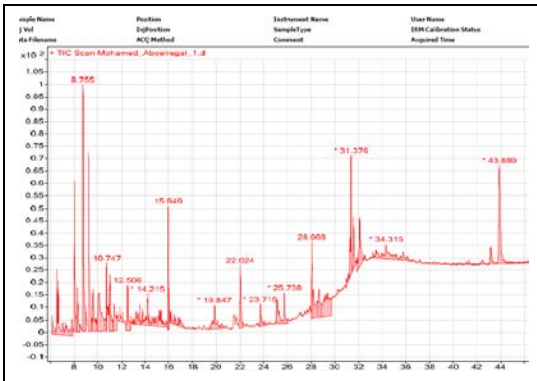
Table 5: MICs of extracts (mg/mL) from selected soft coral against the most previously affected *Penicillium* species [a= concentration of extract (mg/mL), while b= zone, mm].

	MICs (mg/mL)/Fungus					
	P. aurantiogriseum		*P. chrysogenum*		*P. oxalicum*	
Species/extract	Ethanol	Ethyl acetate	Ethanol	Ethyl acetate	Ethanol	Ethyl acetate
S. polydactyla	10[a]	10[a]	10[a]	30[a]	30[a]	30[a]
	9[b]	9[b]	9[b]	9[b]	11[b]	9[b]
S. gracile	50[a]	10[a]	100[a]	10[a]	30[a]	50[a]
	9[b]	13[b]	9[b]	11[b]	11[b]	9[b]
S. glaucum	30[a]	10[a]	10[a]	10[a]	30[a]	50[a]
	11[b]	13[b]	9[b]	12[b]	11[b]	9[b]
S. trocheliophorum	50[a]	10[a]	50[a]	100[a]	30[a]	50[a]
	11[b]	12[b]	9[b]	9[b]	10[b]	10[b]
S. ehrenbergi	30[a]	30[a]	50[a]	50[a]	50[a]	50[a]
	10[b]	9[b]	9[b]	9[b]	9[b]	9[b]
X. macrospiculata	10[a]	30[a]	30[a]	30[a]	100[a]	30[a]
	9[b]	11[b]	10[b]	9[b]	9[b]	10[b]

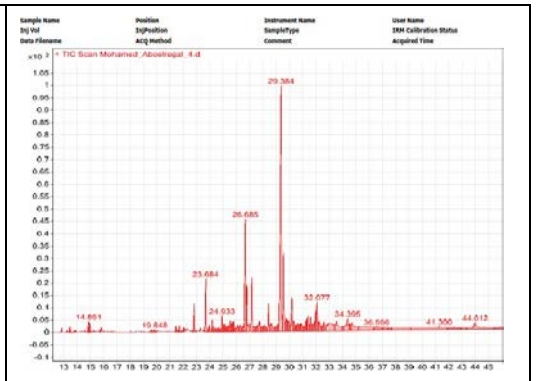
This trail proved the high efficiency of crude extracts under investigation as antifungal agents. Despite of, the crudes of the soft corals that were applied varied in their extract concentration and were not consistent with their antifungal activities; they are very promising based on their AUs and MICs values.

3.4. Composition of potent extracts using GC/MS spectroscopy

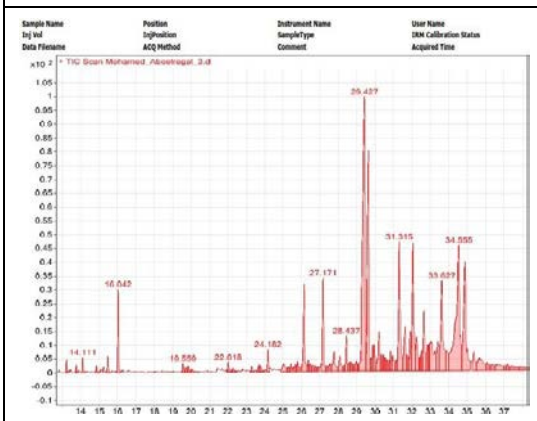
The current study was extended to determine the GC/MS profiles of the most potent extracts (from; *S. polydactyla, S. gracile, S. glaucum, S. trocheliophorum, S. ehrenbergi,* and *X. macrospiculata*) as antifungal agents either ethanolic or ethyl acetate crudes after confirming their high positive records as antifungal agents. The GC/MS chromatogram of them with their retention times (RT), molecular formula, molecular weight, and peak area are presented in Tables 6 & 7 and Figure 2.



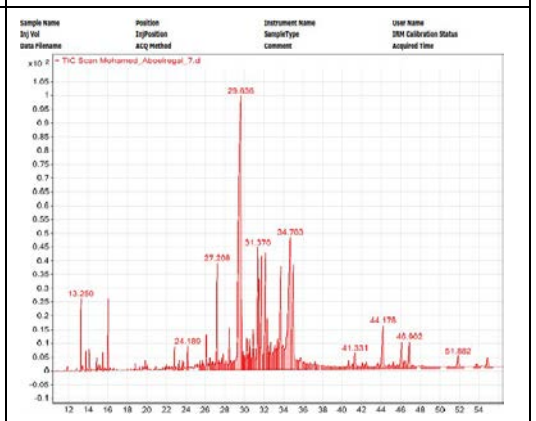
S. polydatyla ethanolic crude extract



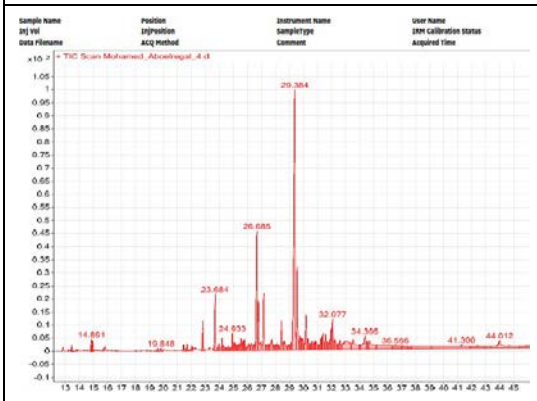
S. polydatyla ethyl acetate crude extract



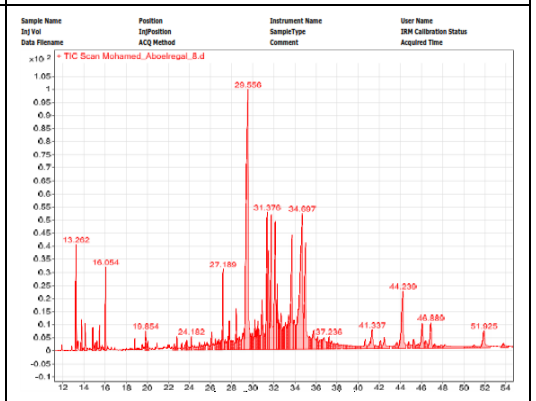
S. ehrenbergi ethanolic crude extract



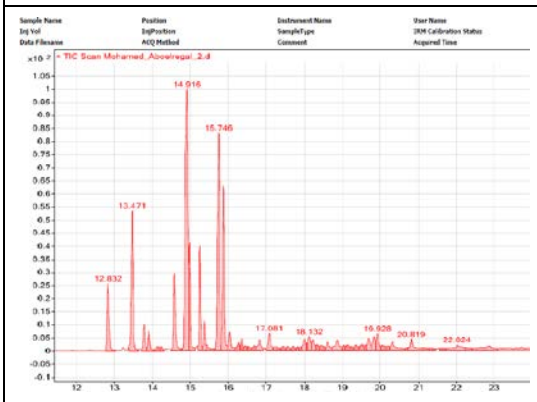
S. ehrenbergi ethyl acetate crude extract



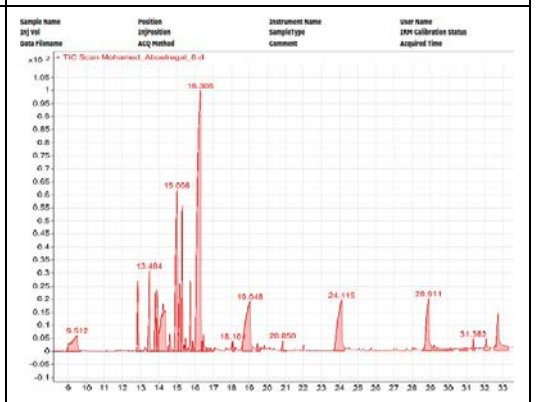
S. tocheliophorum ethanolic crude extract



S. galucum ethyl acetate crude extract



X. macrospoculata ethanolic crude extract



X. macrospoculata ethyl acetate crude extract

Figure 2: Different mass spectra of ethanolic and ethyl acetate of the most potent extracts from soft coral species.

3.4.1. GC/MS profile of major components in the most effective ethanolic extracts

The GC/MS chromatogram of ethanol extract of *S. polydatyla* showed the presence of several active principle compounds. Eleven compounds were identified in this extract. However, the prevailing compounds were (Table 5); 6-octen-1-ol, 3,7-dimethyl-, (R)- (32.7%), 3,7-octadiene-2,6-diol, 2,6-dimethyl- (14.9%), caryophyllene (26.7%), trans-calamenene (42.3%), 8-epi-.gama.-eudesmol (22.1%), 1-heptatriacotanol (32.1%), hexadecanoic acid, ethyl ester (40.2%), 2-hexadecanol (15.3%), 5,8,11,14-Eicosatetraenoic acid, ethyl ester, (all-Z)- (19.0%), diisooctyl phthalate (14.3%), and cholest-5-en-3-ol, 24-propylidene-, (3β)- (36.5%). The GC/MS chromatogram of ethanol extract of *X. macrospoculata* showed the presence of several active principle compounds. Six compounds were identified in this extract. However, the prevailing compounds were; naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)- (54.5%), isolongifolene, 7,8-dehydro-8a-hydroxy- (22.6%), cycloisolongifolene, 8,9-dehydro- (21.2%), -naphthalenemethanol, 1,2,3,4-tetrahydro-8-methyl- (12.5%), murolan-3,9(11)-diene-10-peroxy (27.3%), and nootkaton-11,12-epoxide (32.0%). The GC/MS chromatogram of ethanol extract of *S. ehrenbergi* showed the presence of several active principle compounds. Nine compounds were identified in this extract. However, the prevailing compounds were; cubedol (42.9%), tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethy (12.8%), (Z)6,(Z)9-pentadecadien-1-ol (37.2%), hexadecanoic acid, ethyl ester (55.5%), geranyl-a-terpinene (21.7%), 3,7-cyclodecadien-1-one (23.8%), 3,7-dimethyl-10-(1-methylethylidene)-, (E,E) (9.38%), isoaromadendrene epoxide (50.6%), and 3-oxo-10(14)-epoxyguai-11(13)-en- (34.2%). The GC/MS chromatogram of ethanol extract of *S. tocheliophorum* showed the presence of several active principle compounds. Seven compounds were identified in this extract. However, the prevailing compounds were; isolongifolene, 7,8-dehydro-8a-hydroxy (19.4%), 9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z) (10.5%), thunbergol (41.2%), androstan-17-one, 3-ethyl-3-hydroxy-, (5a) (10.5%), 1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl-6-ethenye-10,14-dimethylene-pentadec-4-enyl)cyclohexane (8.37%), isoaromadendrene epoxide (11.7%), and 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl) (16.5%).

Table 6: GC/MS data of major components in the most effective ethanolic extracts.

Crude extract	Name	RT (min)	Formula	Molecular weight	Probability (%)
S. polydatyla	6-Octen-1-ol, 3,7-dimethyl-, (R)-	8.768	$C_{10}H_{20}O$	156	32.7
	3,7-Octadiene-2,6-diol, 2,6-dimethyl-	10.747	$C_{10}H_{18}O_2$	170	14.9
	Caryophyllene	12.506	$C_{15}H_{24}$	204	26.7
	trans-calamenene	14.203	$C_{15}H_{22}$	202	42.3
	8-epi-.gama.-eudesmol	16.023	$C_{15}H_{26}O$	222	22.1
	1-Heptatriacotanol	19.847	$C_{37}H_{76}O$	536	23.1
	Hexadecanoic acid, ethyl ester	22.036	$C_{18}H_{36}O_2$	284	40.2
	2-Hexadecanol	23.733	$C_{16}H_{34}O$	242	15.3
	5,8,11,14-Eicosatetraenoic acid, ethyl ester, (all-Z)-	28.080	$C_{22}H_{36}O_2$	332	19.0
	Diisooctyl phthalate	31.376	$C_{24}H_{38}O_4$	390	14.3
	Cholest-5-en-3-ol, 24-propylidene-, (3β)-	43.895	$C_{30}H_{50}O$	426	36.5
X. macrospoculata	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	12.832	$C_{15}H_{22}$	202	54.5
	Isolongifolene, 7,8-dehydro-8a-hydroxy-	13.471	$C_{15}H_{24}O$	220	22.6
	Cycloisolongifolene, 8,9-dehydro-	14.016	$C_{15}H_{22}$	202	21.2
	-Naphthalenemethanol, 1,2,3,4-tetrahydro-8-methyl-	15.746	$C_{12}H_{16}O$	176	12.5
	Murolan-3,9(11)-diene-10-peroxy	18.870	$C_{15}H_{24}O_2$	236	27.3
	Nootkaton-11,12-epoxide	22.034	$C_{15}H_{22}O_2$	234	32.0
S. ehrenbergi	Cubedol	14.111	$C_{15}H_{26}O$	222	42.9
	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethy	16.029	$C_{15}H_{24}O$	220	12.8
	(Z)6,(Z)9-Pentadecadien-1-ol	19.559	$C_{15}H_{28}O$	224	37.2
	Hexadecanoic acid, ethyl ester	22.024	$C_{18}H_{36}O_2$	284	55.5
	geranyl-a-terpinene	24.176	$C_{20}H32$	272	21.7
	3,7-Cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (E,E)	27.158	$C_{15}H_{22}O$	218	23.8
	Isoaromadendrene epoxide	29.384	$C_{15}H_{24}O$	220	9.38
	3-Oxo-10(14)-epoxyguai-11(13)-en-6,12-olide	31.296	$C_{15}H_{18}O_4$	262	50.6
	Androstan-17-one, 3-ethyl-3-hydroxy-, (5a)	33.621	$C_{21}H_{34}O_2$	318	34.2
S. tocheliophorum	Isolongifolene, 7,8-dehydro-8a-hydroxy	14.867	$C_{15}H_{24}O$	220	19.4
	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)	19.614	$C_{21}H_{36}O_4$	352	10.5
	Thunbergol	23.678	$C_{20}H_{34}O$	290	41.2
	Androstan-17-one, 3-ethyl-3-hydroxy-, (5a)	24.945	$C_{21}H_{34}O_2$	318	10.5
	1,1,6-trimethyl-3-methylene-2-(3,6,9,13-tetramethyl-6-ethenye-10,14-dimethylene-pentadec-4-enyl)cyclohexane	26.660	$C_{33}H_{56}$	452	8.37
	Isoaromadendrene epoxide	29.366	$C_{15}H_{24}O$	220	11.7
	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)	32.090	$C_{20}H_{34}O_2$	306	16.5

3.4.2. GC/MS profile of major components in the most effective ethyl acetate extracts

The GC/MS chromatogram of ethyl acetate extract of *S. polydatyla* in ethyl acetate showed the presence of several active principle compounds. Three compounds were identified in this extract. However, the prevailing compounds were (Table 6); hexadecanol, 2-methyl (9.42%), dasycarpidan-1-methanol, acetate (ester) (12.1%) and tert-hexadecanethiol (10.5%).

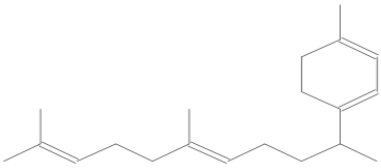
The GC/MS chromatogram of ethyl acetate extract of *X. macrospiculata* showed the presence of several active principle compounds. Four compounds were identified in this extract. However, the prevailing compounds were; naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl), (1S-cis) (27.5%), isolongifolene, 7,8-dehydro-8a-hydroxy, isolongifolene, 7,8-dehydro-8a-hydroxy (20.6%), isoshyobunone (13.0%), and hexaethylene glycol (55.3%). The GC/MS chromatogram of ethyl acetate extract of *S. ehrenbergi* showed the presence of several active principle compounds. Six compounds were identified in this extract. However, the prevailing compounds were; alloaromadendrene (12.7%), geranyl-a-terpinene (12.9%), 3,7-cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (E,E) (13.5%), 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl) (28.9%), 3-oxo-10(14)-epoxyguai-11(13)-en-6,12-olide (12.1%), and campesterol (25.6%). The GC-MS chromatogram of ethyl acetate extract of *S. galucum* showed the presence of several active principle compounds. Six compounds were identified in this extract. The prevailing compounds were; alloaromadendrene (12.2%), tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl (12.8%), 3,7-cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (E,E) (13.0%), 4,8,13-cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl) (28.6%), 3-oxo-10(14)-epoxyguai-11(13)-en-6,12-olide (23.7%), and campesterol.(31.8%).

Table 7: GC/MS data of major components in the most effective ethyl acetate extracts.

Crude extract	Compound name	RT (min)	Formula	Molecular weight	Probability (%)
S. polydatyla	-Hexadecanol, 2-methyl	23.715	$C_{17}H_{36}O$	256	9.42
	Dasycarpidan-1-methanol, acetate (ester)	29.396	$C_{20}H_{26}N_2O_2$	326	12.1
	tert-Hexadecanethiol	32.090	$C_{16}H_{34}S$	258	10.5
X. macrospiculata	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)	13.477	$C_{15}H_{22}$	202	27.5
	Isolongifolene, 7,8-dehydro-8a-hydroxy	15.002	$C_{15}H_{24}O$	220	20.6
	Isoshyobunone	16.275	$C_{15}H_{24}O$	220	13.0
	Hexaethylene glycol	24.084	$C_{12}H_{26}O_7$	282	55.3
S. ehrenbergi	Alloaromadendrene	13.244	$C_{15}H_{24}$	204	12.7
	Geranyl-a-terpinene	24.207	$C_{20}H_{32}$	272	12.9
	3,7-Cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (E,E)	27.201	$C_{15}H_{22}O$	218	13.5
	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)	29.618	$C_{20}H_{34}O_2$	306	28.9
	3-Oxo-10(14)-epoxyguai-11(13)-en-6,12-olide	34.654	$C_{15}H_{18}O_4$	262	12.1
	Campesterol	44.178	$C_{28}H_{48}O$	400	25.6
S. galucum	Alloaromadendrene	13.250	$C_{15}H_{24}$	204	12.2
	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl	6.048	$C_{15}H_{24}O$	220	12.8
	3,7-Cyclodecadien-1-one, 3,7-dimethyl-10-(1-methylethylidene)-, (E,E)	27.165	$C_{15}H_{22}O$	218	13.0
	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)	29.526	$C_{20}H_{34}O_2$	306	28.6
	3-Oxo-10(14)-epoxyguai-11(13)-en-6,12-olide	31.358	$C_{15}H_{18}O_4$	262	23.7
	Campesterol	44.209	$C_{28}H_{48}O$	400	31.8

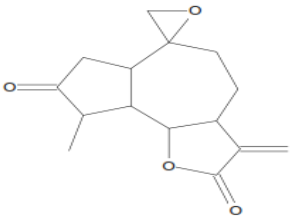
Furthermore, the structures of the most common compounds are illustrated in Figure 3. However, the prevailing compounds were: fatty acids and their derivatives (tetradecanoic acid, ethyl ester and methyl ester of hexadecanoic acid, methyl ester of pentadecanoic acid, methyl ester of octadecenoic acid, methyl ester of tridecanoic acid, Trans-13-octadecenoic acid, methyl ester, and octadecatrienoic acid), terpenoid (Nootkaton-11,12-epoxide, caryophyllene, geranyl-a-terpinene, etc.), steroid (corticosterone, cis-calamenene, etc.), and others.

The previous data revealed that the extracts of current corals differ in their chemical composition besides the potency of the active metabolites. The GC/MS profiles of the potent extracts from either ethanolic or ethyl acetate crudes were obtained, and it was found that the most found compounds were: fatty acids and their derivatives, terpenoid (nootkaton-11,12-epoxide, caryophyllene, geranyl-a-terpinene, etc.), steroid (corticosterone, cis-calamenene, etc.), and others. As the same, several workers employed the GC/MS of *Spongia officinalis* extracts conducting the major constituents were fatty acids and their esters (hexadecanoic acid and octadecanoic acid) which have antimicrobial effect (Abou-Elela *et al.*, 2009; Ibrahim *et al*., 2012).

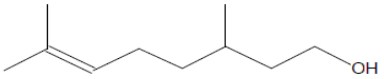


Geranyl-a-terpinene

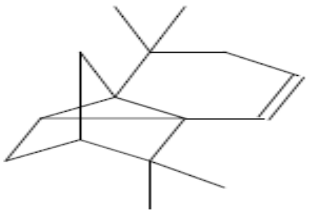
-Hexadecanol, 2-methyl



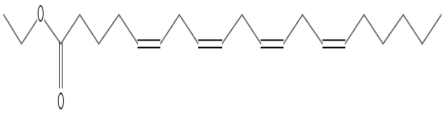
3-Oxo-10(14)-epoxyguai-11(13)-en-6,12-olide



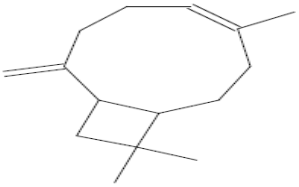
6-Octen-1-ol, 3,7-dimethyl-, (R)-(called also; Citronellol)



Cycloisolongifolene, 8,9-dehydro-

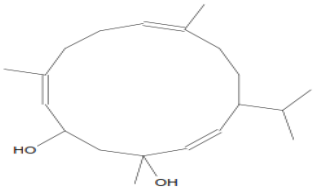


5,8,11,14-Eicosatetraenoic acid, ethyl ester, (all-Z)-



Caryophyllene

Hexadecanoic acid, ethyl ester

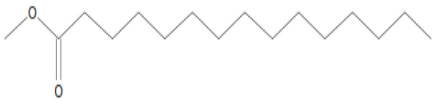


4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)

trans-13-Octadecenoic acid, methyl ester

9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)

Tridecanoic acid, methyl ester



Pentadecanoic acid, methyl ester

Hexadecanoic acid, methyl ester

Figure 3: Structures of the most common compounds in the potent extracts.

Furthermore, the GC/MS patterns conducted by several workers (Ibrahim *et al.*, 2018; Ibrahim *et al.,* 2020a; Ibrahim *et al*., 2020b) were rather in the same direction, which reported that the major constituents of the effective extracts were: fatty acids (tridecanoic acid, hexadecanoic acid, pentadecanoic acid, oleanoic acid, etc.) and their esters, terpenoids, carotenoids, other compounds. These findings also observed most of these bioactive constituents had antimicrobial activities.

Most fatty acids found in the crudes were palmitic acid, oleanolic acid and octadecanoic acid, while fatty acids derivatives were ethyl ester of pentadecanoic, hexadecanoic, tetradecanoic, otadecanoic acids and methyl ester of hexadecanoic, nonadecanoic and tetradecanoic acids. Similarly, many significant constituents such as: palmitic acid, stearic acid, myristic acid, phenols, acetogenins, terpenes, labdane diterpenes, brominated hydroquinones, phlorotannins, and tropodithietic acid, which may produce antibiosis against fungi (Agoramoorthy *et al*., 2007; Balamurugan *et al*., 2013).

In addition, Duh *et al.* (2002) discovered four new diterpenes from the soft coral; *Cespitularia hypotentaculata,* besides six new cadinene sesquiterpenoids, xenitorins A-F, were isolated from the soft coral; *X. puertogalerae.* Moreover, Duh *et al.* (2004) discovered new cytotoxic steroids from both soft corals *Dendronephthya gigantean* and *Lemnalia cervicorni*. Liang and Fang (2006) obtained diterpenes from some *Sarcophyton* species. Sponges are also a good source of unusual sterols. The sulphated and alkaloidal sterols showed potential antimicrobial activity. The existence of terpenoids in sponges is widespread. Most of these compounds exhibit bioactivities. The marine sponge *Tethya aurantia* produces the ether lipids (2*S*)-1-(hexadecyloxy) propane-2, 3-diol, (2*S*)-1-(16 methylheptadecyloxy) propane-2, 3-diol (Ibrahim *et al*., 2017). Moreover, *Spongia officinalis* is well kwon as a source of terpenoids. Antifungal and antibacterial activities have been recorded by the tetracyclic furanoditerpenes isolated from sponge *S. officinalis* (Abou-Elela *et al*., 2009).

3.5. Comparing efficacy of palmitic acid and commercial antifungal drug (Treflucan)

Later, we carried out a trail on the effect of selected fatty acids (especially on palmitic acid as a potent example of bioactive constituents present in extracts) in comparison to antifungal drug (Treflucan) against both *A. niger* and *P. auratiogriseum,,* which they were chosen based on results of MICs in Tables 3 and 4. Data shown in Table 8, revealed that low concentrations (10 and 20 mg/mL) of both palmitic acid and Treflucan did not exhibit any activity against *A. niger*. This appeared because the extracts actually had other constituents not palmitic acid alone. From 50 to 150 mg/mL of both antifungal agents, the AU activity raised from 3.1 to 6.3 against *A. niger.* Only 150 mg/mL showed moderate activities recorded by palmitic acid (AU = 2.3) and Treflucan (AU = 3.1) against *P. auratiogriseum,* respectively. Surprisingly, most of these components, especially palmitic, stearic and tridecanoic acids, methyl ester, had been proven to possess antifungal activities (Rajeswari *et al.,* 2013; Revathi *et al.,* 2014; Kaur *et al.,* 2016).

Table 8: Comparison of (palmitic acid) as representative for the most effective compounds detected in the extracts detected by GC/MS analysis and commercial antifungal drug (Treflucan; Fluconazole).

Agent concentration (mg/mL)	Antifungal activity (AU)/*A. niger*		Antifungal activity (AU)/*P. auratiogriseum*	
	Palmitic acid	Treflucan	Palmitic acid	Treflucan
10	- ve	- ve	- ve	- ve
25	- ve	- ve	- ve	- ve
50	3.1	3.1	- ve	- ve
75	4.0	4.0	- ve	- ve
100	4.0	5.1	- ve	- ve
150	6.3	6.3	2.3	3.1

4. Conclusion

Data obtained during the current study confirmed the idea of the marine invertebrates' usage, particularly soft coral and sponge species, as promising sources for antifungal medication. However, our results proved that both of ethanolic and ethyl acetate extracts of soft corals (*S. polydactyla, S. gracile, S. glaucum, S. trocheliophorum, S. ehrenbergi,* and *X. macrospiculata*) were the most effective extracts that exhibited the most potent antifungal activity (AU). Moreover, the most effective extracts were tested to determine the MICs against the most affected fungi and the lowest effective MIC was 10 mg/mL against several pathogenic fungi. Furthermore, the GC/MS analysis for the most effective extracts showed that fatty acids and their derivatives, terpenoid, steroid, and others were the major constituents. Data also proved the efficacy of palmitic acid as a potent example included in the bioactive constituents present in the extracts, especially when it was compared to Treflucan (a commercial antifungal drug).

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Authors' Contributions

Study Design- Hassan A.H. Ibrahim and EL-Sayed M. El-Morsy; Data Collection- Mohamed S. Amer, Aml Z. Farhat and Mohamed Abu El-Regal; Data Interpretation- Hassan A.H. Ibrahim and EL-Sayed M. El-Morsy; Manuscript Preparation and Literature Search- Marwa T. Mohsien, Mohamed S. Amer and Aml Z. Farhat; Manuscript Revision and Supervision- Hassan A.H. Ibrahim and EL-Sayed M. El-Morsy

Conflict of Interest Disclosure

The above-mentioned manuscript has not been published before and is not under consideration for publication anywhere else. The publication of this article was approved by all authors, as well as by the responsible authorities.

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The Inhibitory Effect of Different Ephedra Plant Extracts on the *Aspergillus flavus* Growth and Aflatoxin B_1 Gene Expression

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Abstract

This study was conducted to investigate the effects of *Ephedra sinica* extracts (ESE) on *Aspergillus flavus* growth, AFB_1 production and *O*-methyltransferase A (*Omt-A*) gene expression. The following various extracts such as Ethanol (EtOH), Ethanol: water (EtOH: H_2O 1:1, v/v), Methanol (MeOH), and Methanol: water (MeOH: H_2O 1:1, v/v) at concentration of 1.0, 2.0, 3.0, 4.0, and 5.0% have been used. AFB_1 production and gene expression were measured using HPLC and real time PCR, respectively. Our findings demonstrated that all ESE has significant inhibition (73.3- 94.6 %) for *Aspergillus flavus* growth based on the dose. Production of AFB_1 was markedly decreased or totally inhibited within range of 10– 99.3% depends on extract type. Supported the effect of ESE at concentrations of 1-5% were suppressed significantly ($P<0.05$) on aflatoxin (*Omt-A*) gene expression. The nucleotides sequence of *Ephedra* plant was submitted in GeneBank under accession number KY310531.1. These outcomes might be used to control the growth of toxigenic fungi and to inhibit the following contamination of feed, food, and agricultural supplies by carcinogenic aflatoxins.

Keywords: Ephedra extracts, Aspergillus, AFB_1 Production, HPLC, gene expression, Omt-A, qRT-PCR.

1. Introduction

AFB_1 is a highly toxic metabolite of food and agricultural resources formed by *Aspergillus species* (Leontopoulos et al., 2003). The disease associated with chronic aflatoxin ingestion is hepatocellular carcinoma (HCC or liver cancer) which is the third-leading cause of cancer death globally according to a World Health Organization report (2008), with about 550,000– 600,000 new cases emerging each year. About 83% of these deaths occur in East Asia and sub-Saharan Afrianca (Parkin et al. 2005, Liu and Wu 2010). Hepatotoxic and hepatocarcinogenic effects of aflatoxins are well known (Wogan, 1999). Aflatoxin exposure has disrupted the synthesis of DNA, chromosome separation, and mitosis progression (Deabes et al., 2012). Aflatoxin B_1 metabolism is divided into 2 phases (Diaz and Murcia 2011). Phase I consists of enzyme mediated oxidation, hydrolysis, reduction reactions. Phase II metabolism consists of conjugation reaction of the compounds modified from the phase I metabolism. Most of the phase I reactions are oxidation or hydroxylation reactions which are mostly catalyzed by cytochrome P450 enzymes. Cytochrome P450, 1A2 and 3A4 enzymes in the liver metabolize aflatoxin into aflatoxin-8,9-epoxide, which then binds to a protein or to DNA and initiates liver carcinoma (HCC) (Wu and Khlangwiset 2010, Eaton and Gallagher 1994). It is evident that Aflatoxin B_1-8,9- epoxide can induce activating mutations in the ras (small GTPase family protein) oncogene in experimental animals. In the presence of water, the epoxide gets hydrolyzed and becomes available to be linked to serum proteins (Groopman et al. 1985). The conjugation step is mostly the detoxification step where aflatoxin undergoes phase II biotransformation (Neal et al. 1998). The resulting aflatoxin conjugates get extracted in the bile. In the deconjugation stage, the bio-transformed aflatoxin gets reabsorbed in the body.

In *Aspergilli*, DNA information is organized into 8 chromosomes, where the genes responsible for the production of aflatoxin are located in the 54th cluster, 80 kb from the chromosome 3 telomere. There are 30 genes in this cluster and its activation is primarily controlled by *aflR* and *aflS*. The aflatoxin gene cluster has been extensively studied in *Aspergillus species*. In *Aspergillus flavus*, the AflR protein binds to at least 17 genes in the AF cluster, resulting in the activation of the enzyme cascade and leading to the production of different AFs. Interestingly, the over-expression of aflR in *Aspergillus flavus* regulates several AF genes, increasing the production of aflatoxin by 50-fold (Montibus et al., 2013). Recently, it has been demonstrated that different inhibitors of aflatoxin production are capable to down-regulate *aflR* expression as the key regulator of the aflatoxin biosynthetic pathway. Disruption of *aflR* completely blocks the expression of the genes in this biosynthetic pathway and aflatoxin/ sterigmatocystin

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production (Caceres et al., 2020). Shimizu and Keller (2001), De Souza et al. (2013) reported that G-protein receptors, cAMP, and PKA have roles in regulating the aflatoxin biosynthetic pathway. In addition, two global regulators of secondary metabolism, *laeA* and *veA* genes regulate the activation of the aflatoxin gene cluster have been documented (Bayram et al., 2008, Sarikaya-Bayram et al., 2015). The LaeA (nuclear protein) contains S-adenosylmethionine binding motif which can activate transcription of a gene cluster in aflatoxin secondary metabolite (Brakhage, 2013). Lae Amethylates histone proteins are associated with cluster for secondary metabolism and make the region accessible to gene transcription. The VeA gene was reported to be essential for the light dependent growth condition. In the absence of light, VeA migrates from cytoplasm into the nucleus to form a complex with LaeA that is essential for both developmental and secondary metabolism (Yin and Keller 2011). Significant decrease in the production of aflatoxin intermediates was reported during the elimination of V*eA* gene from *Aspergillus* strains (Calvo et al., 2004).

On the other hand, the decontamination of mycotoxins by natural products using plant extracts is one of the well-known methods for controlling mycotoxins in foods and feed. Essential oils and aqueous plant extracts have been used as fungal growth inhibitors and to avoid contamination of aflatoxins in food (Abou El-Soud et al., 2012; Ponzilacqua et al., 2018; Deabes et al., 2020). The *Ephedra species* are widely used for their medicinal properties and considered as a potential antimicrobial substitution agent (Ehrlich, 2014; Elhadef et al, 2020a,b). Actually, their organic, aqueous and spice extracts, as well as their essential oils, have shown fungicidal activities (Kuma et al., 2010; Abou El-Soud et al., 2012; Abou El-Soud et al., 2015; Deabes et al., 2020; Elhadef et al, 2020c). So, the present study is conducted to investigate the ability of *Ephedra sinica* extracts to inhibit *Aspergillus flavus* growth followed by investigate its AFB_1 production. Finally, examination of their effect on *O*-methyltransferase A (*Omt-A*) gene expression.

2. Materials and Methods

2.1. Fungal strain, chemicals and Medium.

An *Aspergillus flavus* toxigenic strain (ATCC 28542) was purchased from the Microbial Research Center (MIRCEN), Faculty of Agriculture, Ain-Shams University of Cairo, Egypt. We purchased potato dextrose agar (PDA), yeast extract sucrose (YES) and Na_2SO_3 (Sigma-Aldrich, France). Aflatoxin type B_1 (AFB_1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluroacetic acid (TFA), methanol, acetonitrile of HPLC grade were produced by BDH, Chemicals Ltd., Poole, England. The double distilled water was used by Millipore water purification system (Bedford, M A, USA).

2.2. Preparation of Ephedra sinica and Extracts (ESE)

The *Ephedra* plant was obtained from the mountain in Sinai, Egypt. The aerial parts was washed and then dried in the sunshade at room temperature until all of the aerial parts well dried up. The plant materials were then well powdered using a grinder and put in a container that was well sealed. 10 grams of *Ephedra sinica* powder were extracted by using the solvents Ethanol (EtOH), Pure Ethanol: water (EtOH:H2O 1:1v/v), Pure Methanol (MeOH) and Methanol: water (MeOH:H_2O 1:1 v / v) in a room temperature shaker for 24 hours, then under vacuum filtrated (Rustaiyanet al. 2011 and Deabes et al. 2020). Eventually, the extract was concentrated under vacuum using a rotary evaporator and sterilized using a Nylon Syringe Filter, 0.22 µm.

2.3. Antifungal activity

100 ml of yeast extract sucrose medium were seeded in 500 ml flasks and autoclaved at 120°C for 20 min. The inoculation was added by 1 ml of a spores suspension (10^5 spores) of a aflatoxigenic fungi *A. flavus* ATCC28542 strain with or without of ESE as (1.0 , 2.0,3.0, 4.0 and 5.0%) to the medium. The flasks then were incubated for 14 days at 28°C (Munimbazi and Bullerman 1998). Dry film was used for the determination of AFB1 by (HPLC) according to (Nada et al., 2010, Deabes *et al.,* 2011, Abou El-Soud et al., 2012, Eshak et al., 2013, Abou El-Soud et al., 2015, Deabes *et al.,* 2018). The percentage of inhibition of AFB_1 and mycelia dried weight were calculated using equation: % inhibition = (control - treatment / control) X 100.

2.4. Extraction of Nucleic Acids

For DNA, the DNA was extracted from stem sample of *Ephedra* Plant by using a DNAeasy Plant Mini Kit (QIAGEN, Santa Clarita, CA) according to (Moawed and Ibrahim, 2016). Also, the genomic DNA was isolated from harvested mycelia of *A. flavus* (25 mg) with or without the treatment with (ESE) following instruction of the protocol for DNA Tissue purification mini kit by grinding the liquid N2 frozen tissue in a mortar. Concentration and purity of the total genomic DNA yield were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA), then preserved at -20°C for further use.

For mRNA, total RNA was isolated from selected *A. flavus* strain without treatment (control) or treated with *E. sinica* extracts using RNA Isolation Kit and the mRNA was reverse transcript using cDNA synthesis kit (Roche Applied Science) (Salem et al., 2017; kalill et al., 2018).

2.5. PCR and Sequencing

2.5.1. PCR for rbcL and ITS1

The PCR reaction was carried out as reported by (Ibrahim et al., 2016) in a total volume of 50 µL consisted of the following: 25 µL PCR master mixture (Promega, USA), 20 pcoml of each primer (Invitrogen, USA), 40 ng DNA and ultra-pure water to the final volume. Two primer pairs were designed based on the conservative coding sequences of rbcL and ITS1 as shown in table (1).

Table 1. List of Primer used in this study

Primer	Sequence 5'-3'
rbcL- F	ATGTCACCACAAACAGAGACTAAAGC
rbcL-R	TCGCATGTACCTGCAGTAGC
ITS-1L	CCGCYGAGTAAGTTCGCTCTC
ITS-1R	CCRTTGCCAGATTGCTTCCT
Omt –F	GACCAATACGCCCACACAG
Omt –R	CTTTGGTAGCTGTTTCTCGC

2.5.2. PCR for Aflatoxin B_1 gene

The amplification reactions were carried out in a T100-Bio-Rad Gradient Thermal cycler in mixture of 20 µl total reaction volume consisting of 10 µl of 2 X Go Taq master mix (Promega Corporation, Madison, WI) and 10 pmol of each primer of 50ng DNA and following amplification conditions consisting of initial denaturation at 94°C for 5 min (1 cycle), followed by 35 cycles of 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min, with a final amplification of 72°C for 10 min. Aliquot of amplicon (10 µl) was separated on agarose gel (1.5%) and stained with ethidium bromide (0.1 mg/l) then photographed under Gel Documentation System (Gel DocTM XR+) against size standard consisting of 100 bp DNA Ladder.

2.5.3. PCR Products Purification and Sequencing

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, USA). The sequencing of the PCR product was carried out using the dideoxynucleotide chain termination method with a DNA sequencer (ABI 3730XL, Applied Biosystems) (Microgen, Korea) and a BigDye Terminator version 3.1 Cycle Sequencing RR-100 Kit (Applied Biosystems, USA) following the protocol supplied by the manufacturer. The data obtained were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and aligned using Align Sequences Nucleotide BLAST then submitted to GenBank.

2.5.4. Gene Expression by using qRT-PCR

To assess the copy of the cDNA of *A. flavus* strain treated with *E. sinica* by different solvent extraction EtOH:H_2O(1:1v/v), EtOH and MeOH, MeOH:H_2O (1:1v/v) at concentration 1.0, 2.0, 3.0, 4.0 and 5%**,** a Step One Real-Time PCR System (Applied Biosystem, USA) was used**.** The detection of the expression values of the tested gene was performed according to El-Baz et al., (2016).

2.6. Statistical analysis

Data obtained were analyzed statistically using the Statistical Product and Service Solutions IBM SPSS version 22. Waller–Duncan k-ratio was used for determining degree of significance in the differences among all treated groups (Walter et al 1969). The difference was considered significant based on the probability of $P<0.05$.

3. Results

3.1. Identification of Ephedra sinica plant on molecular level

The conserved regions (rbcl) were amplified, which is primarily required for establishing the DNA barcodes for identification of species. In Ephedra species, it was noticed that good amplicons were obtained with both universal primers for rbc land ITS1.

Both primers resulted in efficient amplification (Figure 1). In this study, samples containing an Ephedra species were sequenced with PCR-amplified fragments of both the rbcL gene and ITS1 regions. Neighbor joining methods were used to establish phylogenetic trees of the rbcL and ITS1 sequences.

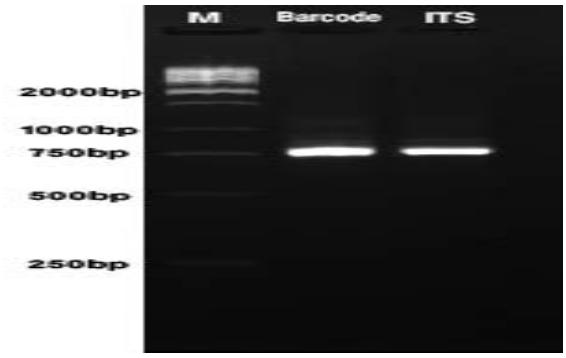

Figure 1. PCR result of DNA barcode regions rbcL and ITS. M; 1kb DNA ladder.

The rbcL sequence phylogenetic tree (Fig. 2) showed that five clades of the ten species formed, *E. sinica*, used in this analysis, have been clustered into *E. Fragile, E. Aphylla & E. Altissima* which suggested a similar genetic relationship between the three species, but *E. sinica* had a distant relationship with the other clade species.

The Basic Local Alignment Tool (BLAST) used for detecting the homology of sequences amplified. The sequence length of rbcL was 750 bp with Ephedra similarly with ITS1, the sequence homology of Ephedra was 99%-100%.

During the present study, it was suggested that the universal primers (rbcL and ITS) for DNA bar coding successful for amplification, identification and discrimination of above mentioned indigenous plant species.

The NJ tree of rbcL DNA sequences correctly placed for the species with the correct family of high statistical support (bootstraps of 99–100%). Due to higher sequence divergences, the rbcL regions can distinguish plant species and simply place these species in the correct family and genus (Fig. 2).

Based on the obtained data found that DNA barcoding can provide accurate plant identification type. The nucleotides sequence of Ephedra plant used in our study was registered in Gene Bank under accession number KY310531.1

Figure 2. Molecular phylogenetic analysis using rbcL for 10 taxa by Neighbor joining (NJ) method.

3.2. Antifungal Activity

3.2.1. Effect of Ephedra sinica extracts (ESE) on growth of Aspergillus flavus ATCC28542 strain and aflatoxin B_1 production in YES medium.

The effect of *Ephedra sinica* extracts was used by different solvents, (MeOH), (MeOH: H_2O 1:1 v/v) (EtOH), (EtOH: H_2O 1:1, v/v) at concentration 1.0, 2.0, 3.0, 4.0 and 5% individual of each showed reduce or inhibition the growth. These results were presented in table (2). Delayed or prevented spore germination, growth inhibition, and decreased of *A. flavus* growth rate at 28 ºC in YES broth medium were observed. Increasing concentration of ephedra plant extracts resulted in a decreasing in total mycelial mass and reducing or preventing production of AFB_1 by *A. flavus* within incubation at 28 ºC for 14 days. Compared with other extracts, MeOH/ H_2O *extract of Ephedra sinica* recorded the lowest effect of inhibition of mycelial growth of *A. flavus*. By increasing concentrations of ephedra extract, the reduction of total mycelia mass was achieved (Figure 2).

Table 2. Percentage of reduction of mycelia dry weight of *Aspergillus flavus* (ATCC 28542) growth (mg/100ml) in YES liquid media treated with *Ephedra sinica* extracts (ESE)

Concentrations of extract (%) **	Type of extract*			
	MeOH:H_2O	MeOH	EtOH:H_2O	EtOH
1	73.3	70.6	80.0	76.0
2	77.3	73.3	82.6	78.6
3	80.0	76.0	86.6	82.6
4	82.6	81.3	89.3	85.3
5	86.6	84.0	94.6	90.6

*Mycelia dry weight in control (75mg/100ml)

Indeed, the growth initiation of *A. flavus* was treated by ephedra solvent extract the observed after 14 days incubation in all flasks. However, growth was slightly inhibited at the lower levels of ephedra solvent extract. The results in Table (3) and Figure (3) were shown the inhibition of AFB_1 production in YES medium. A concentration of ESE by solvents (EtOH: H_2O) 1.0, - 5.0% to YES medium inhibited mycelium and AFB_1 production of *A. flavus* by 80%, 94.6 and 42.8, 98.3% respectively. At the higher concentration 5 % *Ephedra sinica* extract (EtOH: H_2O) in YES average concentration of aflatoxin B_1 was 0.72 µg/100 ml YES medium, and *A. flavus* grew very poorly.

Table 3. Concentrations of AFB_1 (µg/100ml) in liquid media treated with *Ephedra sinica* extracts (n=3)

Concentration of extract (%) **	Type of extract*				
	MeOH:H_2O	MeOH	EtOH:H_2O	EtOH	Mean with extract
[1]	36.96±0.88	38.57±0.63	33.31±0.46	35.06±0.62	35.97±1.6A
2	30.78±1.4	32.5±1.5	22.9±1.3	28.0±0.78	28.56±1.2[B]
3	22.5±0.37	23.25±0.98	16.42±0.52	20.6±0.52	20.68±1.01[C]
4	10.66±0.34	14.9±0.76	2.97±0.3	9.52±0.64	9.52±1.3[D]
5	5.4±0.75	8.5±1.09	0.72±0.25	3.5±0.68	4.52±0.91[E]
Mean with concentration	21.25±3.18[a]	23.54±2.9[b]	15.27±3.3[c]	19.33±3.1[d]	

*AFB_1 in control (43.53µg/100ml)

**Mean values in the row (as a small letter) or Column (as a capital letter) with the same letter are not significant different ($P \leq 0.05$) level.

Data in Table (2) and Figure (3) shown the concentrations of AFB_1 were treated by Four (ESE) in YES medium .Which affected significantly differences to the type of (ESE) and also the concentration of type of their extracts. The extracts of *Ephedra sinica* by different solvent EtOH: H_2O (1:1v/v), EtOH, MeOH, MeOH: H_2O (1:1 v/v) at concentration 1.0, 2.0, 3.0, 4.0 and 5.0% individual of each showed inhibition the AFB_1 production by *A. flavus* in YES medium were determined by HPLC. The percentages of AFB_1 reduction were treated by EtOH: H_2O (42.8, 47.4, 62.3, 93.2 and 98.4 %, respectively) (Figure 4). From the statistical analysis in Table (3), it was found that the effect of mean value with the concentrations of (ESE) for all the treatments EtOH: H_2O (1:1, v/v), EtOH, MeOH and MeOH: H_2O (1:1 v/v), the inhibition percentage of AFB_1 production significantly increased in the YES liquid medium to record 64.9, 55.6, 45.9, and 51.9 %, respectively. Based on the results of current study, it was observed that the positive effect of Ephedra extract against AF production and the AFB_1 biosynthesis by *A.flavus* was limited at all experimented treatments, and the inhibition or prevention depend on the type of solvents extraction and concentration of Ephedra extracts.

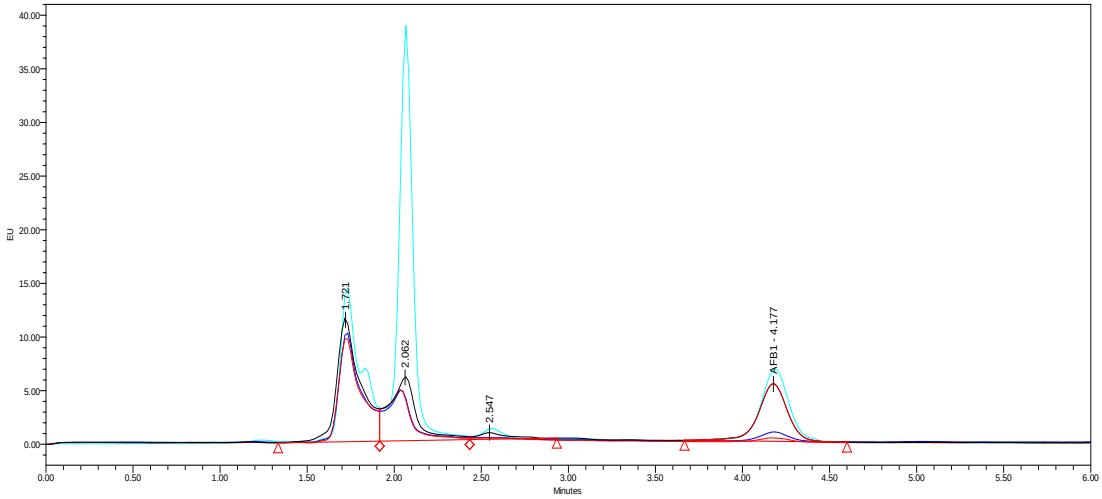

Figure 3. HPLC chromatogram of $AFB_1$with different concentrations of Ephedra (treated by 1-5 % EtOH:H_2O (1:1 v/v)/100 ml media .

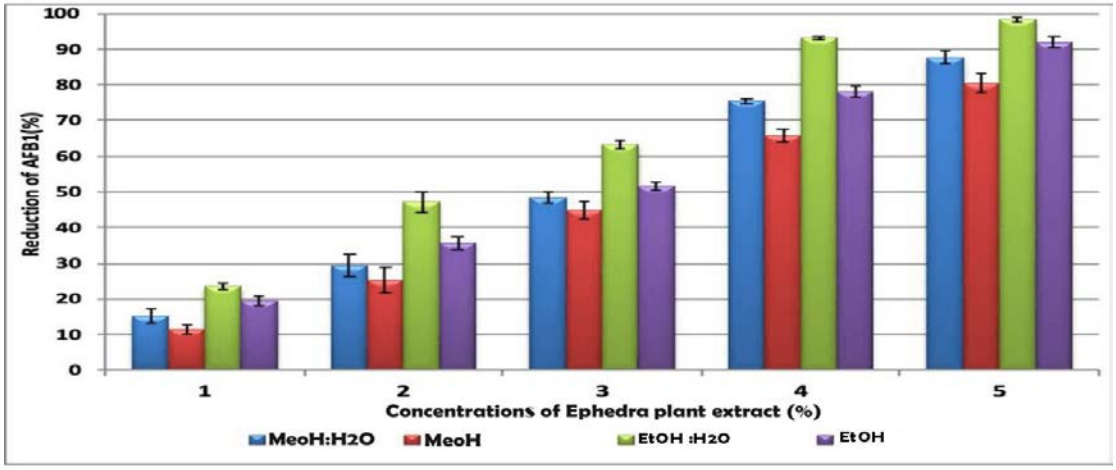

Figure 4 Percentage of reduction AFB_1 in liquid media treated by ESE.

3.3. Polymerase Chain Reaction (PCR) assay.

During the current study, the primers specific for *A. flavus* were designed based on the *omt-A* gene coding for one of the key regulatory enzymes in biosynthesis of AFB_1 (Table 1). Also, they were suitable to detect the fungus. The genomic DNA was extracted from *A. flavus* strain with and without the treatment with aflatoxigenic and then subjected to the PCR using *omt-A* primers. As expected, the DNA fragment with molecular size 300-bp was amplified from tested *A. flavus* isolate. It was found that the assays with *A. flavus* (with and without the treatment with (ESE) yielded a single amplified DNA fragment with expected size (300 bp) (Figure 5).

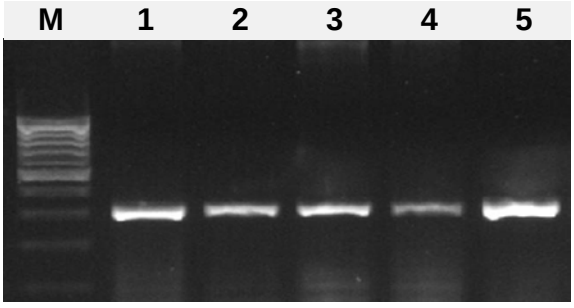

Figure 5. PCR assay using Omt A -primer of *A. flavus*, M .M Gene Ruler 1kb, lane 1; Cont, lane 2; EtOH, Lane 3; MeOH, Lane 4; EtOH:H_2O and Lane 5 MeOH:H_2O.

3.4. The effect ESE on Aspergillus flavus growth and aflatoxin B1 gene (Omt-A) expression on molecular level (quantitative by Real Time-PCR).

The gene (*Omt-A*) expression was analyzed and quantitative by real-time RT-PCR, and the results obtained are summarized in Fig (6). The gene encoding aflatoxin was determined in *A.flavus* fungus cells after treatment with three Ephedra plant extracts; EtOH: H_2O (1:1, v/v), EtOH, and MeOH: H_2O (1:1, v/v).

The results found that treatment of *A. flavus* cells with different plant extracts showed lower expression levels of aflatoxin gene than in control samples. Ethanol (EtOH) extract had a higher influence on aflatoxin gene (*Omt-A*) inhibition (80% decreased significantly compared to the control) followed by EtOH: H2O extract (78% decreased significantly compared to control). On the other hand, MeOH: H2O extract had a lower effect on aflatoxin gene expression than the other two extracts (22% decreased significantly compared to the control).

As for EtOH: H2O and EtOH, they were able to reduce *Omt-A* gene expression Figure (6) by 80 % and 73 % equally and had nearly the same effect on AFB_1 production confirmed by HPLC in (Figure 3) as they reduced it to 84.73 % and 80.76 % respectively in mean values (Table 3, Figures 3& 4).

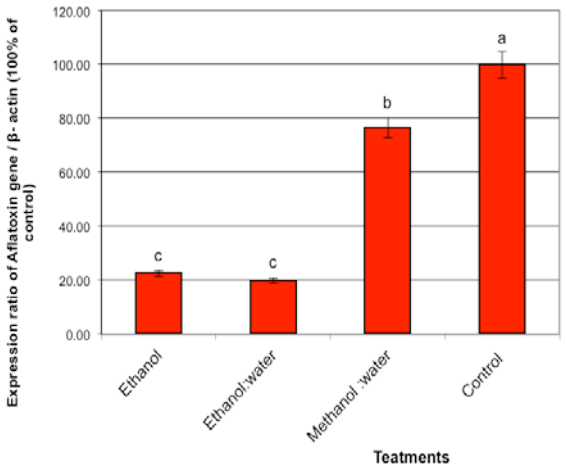

Figure 6. Expression of *Omt_A* gene in *A. flavus* treated with extracts of *Ephedra sinica* by different solvents.

EtOH, EtOH: H_2O (1:1v/v) and MeOHl: H_2O (1:1v/v) at concentrations 1.0, 2.0, 3.0, 4.0 and 5%. Data are presented as mean ± SE. a, b, c followed by different superscripts are significantly different (P≤0.05).

4. Discussion

Mycotoxigenic fungi invade the agriculture commodities and caused Food spoilage and poising causing hazards to humman and animals. So, the researchers looking for untraditional solution for the fungi contaminated food. The Ephedra species have a potent active compounds has the ability to inhibit the fungal growth and have to inhibit the DNA responsible gene of AFB1 produced by *A. flavus*. The current work was start by extraction a potent active compounds from Ephedra stems using different solvents. Then, the DNA were identified using PCR and the nucleotides sequence of Ephedra plant was registries in Gene Bank under accession number KY310531.1. In this respect order (Group et al., 2009) found that the multilocus DNA barcodes have been proposed to be very effective and precise in plant species identification. The rbcL has been proven to be an effective and precise barcode of DNA for plant species identification (Guo et al., 2011). The antifungal of *A. flavus* activity using ESE were studied and noted the efficiency as antifungal against *A. flavus* as well as AFB_1 production. In the same regard, Bagheri et al. (2009), Deabes et al. (2020) found that the Ephedra have antifungal effect. Based on inhibition of growth of *A. flavus* and AFB_1 production due to the potent active compounds such as alkaloids, phenols, glycosides, steroids, coumarins and tannins (Masako, et al., 1989; Ebana, et al., 1991, Deabes et al., 2020).

The obtained data demonstrates that the antifungal effect of ESE can regulate the cellular effects of aflatoxins, and this may be due to the aromatic organic compounds of spices or medicinal plants that can control the formation of secondary metabolites aflatoxins (Chatterjee 1990). Ephdra's antimicrobial effect was related to Cis-314-methanoproline (Caveney et al., 2001) and Heptadecane (Bagheri et al., 2009). Linked to Ephedra's antimicrobial activity, the study by Deabes et al. (2020) found that the extraction of *Ephedra sinica* using EtOH: H_2O solvent was established to be a valuable source for natural polyphenols and alkaloids against pathogenic (*K. pneumoniae, E. cloacae, S. marcescens, E. coli, A. flavus, A. ochraceus, A. niger* and Coxsackie B3 virus).

Due to flavonoid compounds including p-coumaric acid and quercetin in ESE causing on both growth inhibitor and AFB_1 producer by *A. flavus* and *A. parasiticus* (Aziz et al., 1998 ; Kim et al., 2004 , Deabes et al., 2020) .

Most AF biosynthesis inhibitors operate on one of three levels: altering the physiological environment or other signaling inputs perceived by the fungus, interfering with signal transduction and gene expression regulatory networks upstream of AF biosynthesis, or blocking enzymatic activity of a biosynthetic enzyme (Holmes et al., 2008). Several researchers studied the anti-microbial and anti-fungal properties of the species Ephedra. In the another studies, they analysis of plant Ephedra extracts or essential oil (EO) has shown to inhibit *A. parasiticus* or *A. flavus* growth. However, the fungal growth significantly inhibited at the maximum concentration (1000 µg/ml) (Bagheri-Gavkosh et al., 2009; Deabes et al., 2020). Ozdemir et al. (2004) reported that two heptadecane and tetradecane derivatives isolated from *Spirulina platensis* EO had antimicrobial effects on a broad range of microorganisms. Different studies suggest the ability of plant extracts and EOs to inhibit aflatoxigenic fungal growth and AF production. In agreement with our results, organic solvents extract of aerial parts and roots of Ephedra plant were able to inhibit *A. parasiticus* growth and AFB_1 production (Bagheri-Gavkosh et al., 2009). Some phenolic compounds such as acetosyringone, and syringaldehyde can inhibit aflatoxin biosynthesis in *A. flavus* efficiently (Hua et al., 1999). There are some studies on using plant extracts such as afoetida, turmeric and *Azadirachta indica* leaf extract to prevent aflatoxin production (Ghewande and Nagaraj, 1987). Eleven potent active compounds as antioxidant agents and anticancer were detected in ethanol/ water extract of *Saussurea costus* root, which used to mitigate the oxidative stress (Deabes et al., 2021). On the other hand, Holmes et al., (2008) found that norsolorinic acid (NOR), is stable intermediate in the AF biosynthetic pathway. The findings by Nazareth et al. (2020) indicate that allyl isothiocyanate may be used as a fumigant to prevent *A. flavus* growth and AFB_1 development, further confirming transcriptional alteration of AFB_1 genes.

5. Conclusions

Ephedra sinica extracts showed an antifungal activity and prevented the growth of A. *flavus* cultured on YES medium. EtOH/ H_2O extract was the most effective extract to control the biosynthesis of AFB_1 and *A. flavus* regulatory *Omt-A* gene, in combination with a drastic reduction in AFB_1 production. It is worthy to mention that the obtained findings mentioned that ephedra plant extracts have a positively potent effect on the growth and development of aflatoxigenic fungi in food, feed, and agricultural supplies by carcinogenic aflatoxins.

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Bioactivity of Four Nigerian Wild Mushrooms against Some Typed Clinical Isolates

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Abstract

This study aimed at investigating the antimicrobial activity of some wild Nigerian mushrooms against selected typed clinical isolates. We collected wild mushrooms from an integrated organic farm in Ilesa, Southwest Nigeria. Crude methanolic extracts of *Lentinus squarrosulus* Mont., *Termitomyces robustus* (Beeli) R. Heim, *Trametes ochracea* (Pers.) Gilb. & Ryvarden and *Xylaria hypoxylon* (L.) Grev. were screened singly and in different combinations for bioactivity against the selected bacterial and yeast isolates. The minimum inhibitory concentration (MIC) and chemical constituents of the extracts were studied following standard procedures. Overall, we obtained a total of 16 mushrooms belonging to 14 genera. The extracts showed varied clearance zones against at least one of the eight bacteria, and one yeast when applied singly with the antimicrobial inhibitory zone ranging from 7.2 mm to 20.0 mm in *Staphylococcus aureus* (*T. ochracea* extract) and *Pseudomonas aeruginosa* (*L. squarrosulus* extract) respectively. Furthermore, the MIC ranged from 2.09 to 16.75 mg/mL. When combined, the blends were active against some Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis*) but inactive against the Gram-positive bacteria and yeast. Except for *X. hypoxylon*, other extracts contained saponins, tannins and terpenoids. Our findings revealed that the wild mushrooms are potential antimicrobial agents against the tested isolates.

Keywords: Wild mushrooms; Bioactivity; Clinical isolates; Minimum inhibitory concentration; Bacteria; Yeast

1. Introduction

Mushrooms are valuable food source and nutraceuticals owing to their rich nutrient and preventive capability of various ailments (Valverde *et al*., 2015; Roy *et al*., 2016). In nature, they are found all-year-round but more abundantly during the wet season in the terrestrial or ligneous habitats (Adeniyi *et al*., 2018a). Macrofungi thrive on a variety of substrates, especially those rich in lignin, cellulose and organic matter. Thus, they play a significant role in the terrestrial ecosystem as biodegraders (Adebiyi and Yakubu, 2016; Adeniyi *et al*., 2018a).

In alternative medicine, mushrooms are famous for their therapeutic value against ailments such as rheumatism, kwashiorkor, obesity, diarrhoea, and as a purgative (Apetorgbor *et al*., 2005; Ejelonu *et al*., 2013). Earlier studies revealed the anticholesterol, antitumor, antimicrobial, antiviral, antineoplastic, antimutagenic, antioxidant, antilipidemic, antidiabetic, antihyperglycaemic, antihypotensive, antiparasitic, anti-inflammatory, hepatoprotective, hypocholesterolemic, immunomodulatory and anti-ageing properties of mushrooms (Iwalokun *et al*., 2007; Patel *et al*., 2012; Sevindik, 2019; Mushtaq *et al*., 2020). However, inadequate scientific investigations, the dearth of clinical trials, and lack of data to validate the evidence limited their acceptance as drugs in modern-day medicine (Sullivan *et al*., 2006).

The increasing failure of chemotherapeutics recently mandated the quest for newer and less expensive antimicrobials effective against disease-causing microorganisms (Kotra and Mobashery, 1998; Thomson and Moland, 2000; Saki *et al*., 2020; Sevindik, 2020). The search for inexpensive but potent antimicrobial is essential for the low- and middle-income African countries including Nigeria, that are currently hit by the menace of multidrug resistance and infectious diseases (Okeke *et al*., 2005). Fortunately, mushrooms, which commonly grow in the wild in Nigeria are getting the scientists' attention for possible development into novel drugs (Alves *et al*., 2012; Roy *et al*., 2016; Khatua *et al*., 2017; Krupodorova and Sevindik, 2020).

About 140,000 mushroom species exist globally, but a small percentage has been investigated for their therapeutic property and pharmacological screening (Wasser, 2002). A recent study, however, documented 158 mushroom species identified as potential antibiotic sources (Shen *et al*., 2017).

Unfortunately, out of the 172 wild mushrooms reported so far in Nigeria, the most populous black African country, only 26 have been screened for their antimicrobial and pharmacological activities. These include *Auricularia polytricha* (Mont.) Sacc., *Boletus* sp., *Coprinellus*

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micaceus (Bull.) Vilgalys, Hopple & Jacq. Johnson, *Corilopsis occidentalis* (Klotzsch) Murrill, *Daedalea elegans* Spreng, *D. quercina* (L.) Pers., *Daldinia concentrica* sensu auct. NZ, *Flammulina* sp., *Ganoderma lucidum* sensu auct. asiatic., *Lentinus squarrosulus* Mont., *Lenzite quercina* (L.) P.Karst., *Lycoperdon giganteum* Batsch, *L. pusillum* sensu auct. mult., *Marasmius oreades* (Bolton) Fr., *Pleurotus ostreatus sensu Cooke, P. tuber-regium* (Fr.) Singer, *Psathyrella atroumbonata* Pegler, *Psalliota campestris* (L.) Quél., *Schizophyllum commune* Fr., *Termitomyces robustus* (Beeli) R. Heim, *Trametes elegans* (Spreng.) Fr., *T. versicolor* (L.) Lloyd, *Trichaptum* sp., *Tricholoma lobayensis* R. Heim, *T. nudum* (Bull.) P. Kumm and *Volvariella volvacea* (Bull.) Singer from different regions such as Ebonyi (Udu-Ibiam *et al*., 2014; Udu-Ibiam *et al*., 2015), Ekiti (David *et al*., 2012), Abuja (Etim *et al*., 2014), Kogi (Ayodele and Idoko, 2011), Ondo (Ogidi *et al*., 2015), Oyo (Jonathan and Fasidi, 2003; Gbolagade and Fasidi, 2005; Awala and Oyetayo, 2015) and Uyo (Etim *et al*., 2012).

Our previous investigation on the biodiversity of wild mushrooms in ENPOST integrated organic farm, Ilesa, Osun State Southwest Nigeria, had 151 mushroom species documented (Adeniyi *et al*., 2018a). To our knowledge, none of the species has undergone screening for antimicrobial potential, and it is in this light that the current study aimed at elucidating some wild mushrooms from the farm for possible bioactivity.

2. Materials and Methods

2.1. 2.1 Description of the Study Area

Environmental Pollution Science and Technology (ENPOST) integrated organic farm is located between Latitude 4°42′30′E to 4°42′45′′E and longitude 7°36′55′′N to 7°37′10′′N, Ilesa, Osun State, Southwest Nigeria. The farm which is on a large expanse, about 10 hectares of land was established to address the challenges of environmental pollution, food insecurity, agroforestry/biodiversity destruction, and provide research opportunities (Adeniyi *et al*., 2018a).

2.2. Mushroom Collection and Identification

Fresh mushroom fruiting bodies were collected during May and October 2017. Samples were gently placed in paper bags and immediately transported to the laboratory for identification using standard keys (Odeyemi and Adeniyi, 2015).

2.3. Habitat and Substrate Classification of Mushroom Samples

The habitats and substrates of the mushrooms were differentiated alongside sample collection. Samples obtained were either classified as ligneous or terrestrial habitat whereas, substrate classification was based upon woody or soil-like material.

2.4. Preparation of Crude Extracts

The mushroom samples were oven-dried at 40°C for 1 – 5 d, ground into powder using an electric blender, sieved through 160 mesh and preserved in an airtight plastic container prior extraction. After pulverization, four samples were selected for further analysis. Exactly 50 g of the mushroom powder was extracted by soaking in 200 mL of 70 % methanol for 3 d with continuous agitation and thereafter filtered using a muslin cloth and Whatman no. 1 paper. Additionally, the residue was extracted twice using the same solvent, evaporated at 65°C, and the resultant semisolid extract was freeze-dried and kept at 4°C before use.

2.5. Test Organisms

All isolates used in this study were sourced from the National Institute of Medical Research (NIMR), Yaba, Lagos State, Nigeria. They included five Gram-negative bacteria [(*Escherichia coli* (ATCC 25900), *Klebsiella pneumoniae* (ATCC 43816), *Proteus mirabilis* (ATCC 7002), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella typhimurium* (ATCC 14028)], three Gram-positive bacteria [*Bacillus subtilis* (NCTC 8263), *Corynebacterium diphtheriae* (ATCC 13812), *Staphylococcus aureus* (NCTC 6571)] and a yeast, *Candida albicans* (ATCC 10231).

2.6. Antimicrobial Assay

Twenty-four-hour (24 h) old bacterial and 48 h old yeast broth cultures were washed in physiological saline thrice and standardized to 0.5 McFarland standard having approximately 10^8 CFU/mL for bacteria and 10^7 CFU/mL for *C. albicans*. Lyophilized extracts were dissolved in 3 % dimethylsulfoxide (DMSO) to a concentration of 67 mg/mL, sterilized by passing through a membrane filter (0.22 µm pore size), and kept in amber bottles at 4°C. The antimicrobial assay was carried out using the standardized agar well diffusion method (CLSI, 2018). Exactly 100 µL of 0.5 McFarland standardized culture was spread plated on Mueller Hinton agar (Oxoid, UK) using a sterile swab and allowed to dry. A sterile 7 mm cork borer was used to create wells and 50 µl (67 mg/mL) of mushroom crude extracts were added to the holes. After incubating bacteria at 37°C for 24 h and yeast at 27°C for 48 h, antimicrobial activities were determined by measuring the diameter (in millimetres) of inhibition. Negative control was pure DMSO solvent, whereas positive controls were gentamicin (30 mg) for bacteria and fluconazole (25 µg) for yeast.

2.7. Combination Effect of Extracts on the Test Isolates

The same antimicrobial assay previously described was employed. The synergistic, antagonistic, indifference and additive effects of the extracts were determined in dual, triple and quadruple combinations. Each blend consisted of 67 mg/mL of individual crude extract.

2.8. Determination of Minimum Inhibitory Concentration of the Extracts

The minimum inhibitory concentration (MIC) was determined by macro-broth dilution technique as specified (CLSI, 2018). Double-fold dilution of 67 mg/mL extract was prepared in Muller Hinton broth to obtain 6 different concentrations (34.50, 16.75, 8.38, 4.19, 2.09, 1.05 mg/mL). Each dilution was seeded with 100 µL of the standardized suspension of the test organisms and incubated under standard condition. The lowest concentration that showed no visible growth was considered as MIC.

2.9. Screening of the Mushroom Extracts for Chemical Constituents

The mushroom extracts were qualitatively screened for saponins, tannins, terpenoids and anthraquinones as described (Sofowora, 1993). Briefly, saponins were detected by adding 5 mL distilled water to 5 ml of the extract, with vigorous shaking and warming. The formation of stable foam indicates the presence of saponins. In tannins, 3 mL of the extract was added to 3 mL 10 % $FeCl_3$. A blue/black colouration suggests tannins. Furthermore, 5 mL of the extract mixed with 2 mL chloroform and 3 mL concentrated H_2SO_4 was gently poured into the tube. A reddish-brown colouration at interface indicates the presence of terpenoids. Also, 0.5 g of extract was boiled in 10 % HCl and filtered when hot. To the filtrate, 2 mL of chloroform and 10 % NH_3 solution was added. Development of the pink colour in the aqueous layer indicates anthraquinones.

3. Results and Discussion

3.1. Mushrooms Species Obtained at the Site of Study

Mushrooms are non-timber forest products and have served as food, medicine, enzymes, and are also an important source of earnings for people in different parts of the world (Boa, 2004). However, human activities such as deforestation, bush burning, application of pesticides and herbicides, urbanization and climate change have resulted in their gradual disappearance in the wild (Adeniyi *et al*., 2018a).

In the present investigation, a total of 16 mushrooms belonging to 14 genera were obtained, of which eleven species were collected in May, one species in October and four species during both months (Table 1). Representative pictures are in Figure 1. Previous studies in India (Singha *et al*., 2017), Mexico (Álvarez-Farias *et al*., 2016), Italy (Leonardi *et al*., 2017), Ethiopia (Sitotaw *et al*., 2015) and Nigeria (Adeniyi *et al*., 2018a,b; Buba *et al*., 2018), have recorded related mushroom species, with some even at higher frequencies.

Table 1. Sampling months and mushrooms species collected.

Sampling month	Mushroom
May 2017	*Cantharellus cibarius* Fr.
	Collybia plicatilis (Curtis) Fr.
	Clitopilus prunulus (Scop.) P. Kumm.
	Collybia sp.
	Gloeophyllum sepiarium (Wulfen) P. Karst.
	Hydnellum peckii Banker
	Mycena acicula (Schaeff.) P. Kumm.
	Mycena inclinata (Fr.) Quél.
	Pleurotus lignatilis (Pers.) P. Kumm.
	Stereum hirsutum (Wild.) Pers.
	Tricholoma inocybeoides A. Pearson
October 2017	*Termitomyces robustus* (Beeli) R. Heim
May and October 2017	*Ganoderma resinaceum* Boud.
	Lentinus squarrosulus Mont.
	Trametes ochracea (Pers.) Gilb. & Ryvarden
	Xylaria hypoxylon (L.) Grev.

Figure 1. Representative pictures of mushrooms obtained from the site of study. (a) *Hydnellum peckii* (b) *Lentinus squarrosulus* (c) *Mycena inclinata* (d) *Termitomyces robustus* (e) *Trametes ochracea* (f) *Xylaria hypoxylon*.

3.2. Habitat and Substrate of Mushroom Samples

Mushrooms have a wide ecological range and can grow in both coniferous and broadleaf forests (Leonardi *et al*., 2017; Sevindik *et al*., 2018). While we obtained eleven of our mushrooms from ligneous habitat, the remaining five came from the terrestrial counterpart. This observation concurs with an earlier report (Buba *et al*., 2018). The number of mushroom species found on decaying ligneous substrates was in the order: 3 (18.75 %) each of mango and palm, 2 (12.5 %) each of kola nut and unidentified trunk logs and bamboo leaves 1 (6.25 %), whereas on terrestrial substrates, were soil debris 4 (25 %) and termite mound 1 (6.25 %) (Figure 2). Additionally, the mushrooms were

found to be habitat- and substrate-specific, as described elsewhere (Adeniyi *et al*., 2018a).

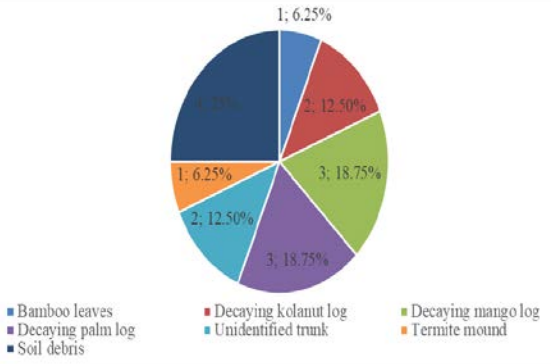

Figure 2. The occurrence of the mushroom species on growth substrates.

3.3. Antibacterial and Antifungal Screening

All the extracts screened had varied clearance zones against at least one of the eight bacteria, and the yeast except *X. hypoxylon*. Generally, the inhibitions of the extracts against the test isolates were in the order *T. ochracea* > *T. robustus* > *X. hypoxylon* > *L. squarrosulus* (Table 2). Among the isolates tested, *P. aeruginosa* (*L. squarrosulus* extract) had the highest inhibition (20 mm), whereas *S. aureus* (*T. ochraceus* extract) had the lowest (7.2 mm) (Table 2). Our observation tallies with Chowdhury *et al*. (2015) whose report ranged between 7.0 ± 0.2 and 30.0 ± 0.3 mm. The production of slime and capsule in microorganisms are responsible for variability in the potency of the extracts (Awala and Oyetayo, 2015; Murray *et al*., 2013).

Generally, not all inhibition zones observed in an *in-vitro* sensitivity test are considered sensitive (CLSI, 2018). According to Chowdhury *et al*. (2015), mushroom crude extracts are highly effective when the clearance diameter is greater than 10 mm. In the current investigation, *P. aeruginosa* and *C. albicans* were sensitive to *L. squarrosulus*; *E. coli*, *K. pneumoniae*, *S. typhimurium* and *C. albicans* to *T. robustus*; *E. coli*, *K. pneumoniae*, *B. subtilis*, *C. diphtheria*, *S. aureus* and *C. albicans* to *T. ochracea*; *E. coli* and *B. subtilis* to *X. hypoxylon* (Table 2). Generally, Gram-positive bacteria are more susceptible to different medicinal compounds than Gram-negative because of the porous peptidoglycan layer and single lipid bilayer in Gram-positive bacteria (Khatua *et al*., 2017). In contrast, the current study observed higher susceptibilities in Gram-negative to the different extracts screened (Table 2). Our finding agrees with Awala and Oyetayo (2015) who also reported low resistance in Gram-positive bacteria in the presence of *Trametes elegans* extract, suggesting that the antimicrobial activities of the extracts may not be cell wall-related.

Broad-spectrum antimicrobials have played an invaluable role in treating bacterial infections and saved lives in situations where early diagnosis and identification of infectious diseases' causative agents is not possible (Melander *et al*., 2018). This current study reveals the broad-spectrum activities of *T. robustus*, *T. ochracea* and *X. hypoxylon* extracts against bacteria and yeast screened (Table 2). A previous study (Sharma *et al*., 2015) reported the broad-spectrum nature of *Agaricus bisporus*. One strategy being highlighted in the fight against bacteria resistance is the development of narrow-spectrum antimicrobials that are either genus or species-specific (Melander *et al*., 2018). Our work reveals the species-specificity of *L. squarrosulus* extract and thus, can be a potential drug for *P. aeruginosa* infections. The range of inhibition by the standard drugs was between 18.2 and 26.0 mm (Table 2).

Table 2. Diameter of inhibition of crude extracts against test microorganisms.

Mushroom extract	Gram-negative bacteria					Gram-positive bacteria			Yeast
	EC (mm)	KP (mm)	PA (mm)	PM (mm)	ST (mm)	BS (mm)	CD (mm)	SA (mm)	CA (mm)
L. squarrosulus	0.0±0.0	0.0±0.0	20.0±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	14.1±0.1
T. robustus	10.2±0.2	12.0±0.1	0.0±0.0	0.0±0.0	14.0±0.1	0.0±0.0	0.0±0.0	0.0±0.0	19.2±0.1
T. ochracea	10.1±0.1	14.2±0.2	0.0±0.0	8.1±0.1	9.2±0.2	11.1±0.1	10.0±0.0	7.2±0.2	17.0±0.1
X. hypoxylon	15.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	19.0±0.0	14.0±0.3	0.0±0.0	0.0±0.0	0.0±0.0
Negative control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Standard drug	19.0±0.1	21.0±0.1	21.1±0.1	18.2±0.1	26.0±0.0	20.1±0.2	19.0±0.1	18.1±0.0	22.8±0.3

Legend**:** EC – *E. coli*; KP – *K. pneumoniae*; PA – *P. aeruginosa*; PM – *P. mirabilis*; ST – *S. typhimurium*; BS – *B. subtilis*; CD – *C. diphtheria*; SA – *S. aureus*; CA – *C. albicans*.

3.4. Effect of Different Extract Combinations on the Test Isolates

Combination therapy has been envisaged to be an effective strategy in treating complex infections (Xu *et al.,* 2018) and are more superior compared to single drug dosage (Vakil and, Trappe, 2019). Generally, all the extract blends were resistant to 2 Gram-negative bacteria, *K. pneumoniae* and *S. typhimurium*, all the Gram-positive bacteria and the fungus (Table 3). It is possible that the effects of active ingredient which may be present in some of the extracts were concealed by other compounds in the mixture and thus suggests antagonism between the individual extracts in combination. Usually, drug antagonism is often undesirable but could be a useful selective factor for drug-resistant mutations (Chait *et al*., 2007). Furthermore, the extract composites were sensitive to at least one of *E. coli*, *P. aeruginosa* and *P. mirabilis* with different relationships. Except for combination *L. squarrosulus* and *X. hypoxylon* which was antagonistic, other active mixtures were indifferent to *E. coli*. Likewise, an indifferent relationship was observed against *P. aeruginosa* albeit synergistic in *T. ochracea* and *T. robustus* blend. Interestingly, the interactions between A, B and G against *P. mirabilis* were synergistic (Table 3). In drug production, synergistic interaction is preferable due to its effectiveness (Xu *et al*., 2018).

Table 3. Sensitivity pattern of the different crude extract combinations.

Extract combinations	Gram-negative					Gram-positive			Fungus
	EC (mm)	KP (mm)	PA (mm)	PM (mm)	ST (mm)	BS (mm)	CD (mm)	SA (mm)	CA (mm)
A	15.1±0.1	0.0±0.0	19.1±0.0	10.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
B	14.2±0.2	0.0±0.0	13.3±0.3	10.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
C	12.1±0.0	0.0±0.0	0.0±0.0	11.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
D	13.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
F	10.2±0.2	0.0±0.0	0.0±0.0	12.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
G	8.1±0.0	0.0±0.0	12.0±0.1	11.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
H	11.4±0.2	0.0±0.0	0.0±0.0	12.0±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
I	0.0±0.0	0.0±0.0	0.0±0.0	10.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
J	0.0±0.0	0.0±0.0	0.0±0.0	12.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Negative control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Standard drug	19.0±0.1	21.0±0.1	21.1±0.1	18.2±0.1	26.0±0.0	20.1±0.2	19.0±0.1	18.1±0.0	22.8±0.3

Legend: EC – *E. coli*; KP – *K. pneumoniae*; PA – *P. aeruginosa*; PM – *P. mirabilis*; ST – *S. typhimurium*; BS – *B. subtilis*; CD – *C. diptheriae*; SA – *S. aureus*; CA – *C. albicans*

A – *T. ochracea* + *T. robustus*; B – *T. ochracea* + *L. squarrosulus*; C – *T. ochracea* + *X. hypoxylon*; D - *T. robustus* + *L. squarrosulus*; E - *T. robustus* + *X. hypoxylon*; F - *L. squarrosulus* + *X. hypoxylon*; G - *T. ochracea* + *T. robustus* + *L. squarrosulus*; H - *T. ochracea* + *T. robustus* + *X. hypoxylon*; I - *T. ochracea* + *L. squarrosulus* + *X. hypoxylon*; J - *T. ochracea* + *T. robustus* + *L. squarrosulus* + *X. hypoxylon*.

3.5. MIC of the Mushroom Extracts

In this investigation, MIC ranged between 2.09 and 16.75 mg/mL (Table 4). While the lowest value (2.09 mg/mL) was recorded in *E. coli* (*T. robustus*), *S. typhimurium* (*T. ochracea*) and *C. diphtheriae* (*T. ochracea*), the highest (16.75 mg/mL) was obtained in *C. albicans* for *L. squarrosulus* and *T. ochracea* extracts (Table 4). Our observation contradicts the previous report (Chowdhury *et al*., 2015) on low MIC for their fungal species investigated.

Table 4. MIC values (mg/mL) of the mushroom extracts against the test isolates.

Mushroom	MIC concentration (mg/mL)								
	EC	KP	PA	PM	ST	BS	CD	SA	CA
L. squarrosulus	ND	ND	8.38	ND	ND	ND	ND	ND	16.75
T. robustus	2.09	4.19	ND	ND	4.19	ND	ND	ND	8.38
T. ochracea	4.19	8.38	ND	4.19	2.09	4.19	2.09	8.38	16.75
Xylaria hypoxylon	4.19	ND	ND	ND	8.38	8.38	ND	ND	ND

Legend**:** EC – *E. coli*; KP – *K. pneumoniae*; PA – *P. aeruginosa*; PM – *P. mirabilis*; ST – *S. typhimurium*; BS – *B. subtilis*; CD – *C. diphtheriae*; SA – *S. aureus*; CA – *C. albicans*; ND – Not determined.

3.6. Chemical Components of the Mushroom Extracts

Mushrooms are rich in phytochemicals such as polyketides, steroids, terpenes, ceramides, glycoproteins, proteoglycans, polysaccharides and phenols (Chowdhury *et al*., 2015). Our findings frequently detected saponins, terpenoids and tannins in *L. squarrosulus, T. robustus* and *T. ochracea* extracts (Table 5). This finding tallies with Gbolagade and Fasidi (2005) and Anyanwu *et al*. (2016) who had similar observations for *Trametes elegans* (Spreng.) Fr. and *Pleurotus tuber-regium* (Fr.) Singer sclerotium. The absence of saponins, terpenoids and tannins in *X. hypoxylon* (Table 5) is contrary to the evidence of Jang *et al*. (2009) and Elias *et al*. (2018) that genus *Xylaria* contains a diversity of bioactive substances. Different ecological locations, age of mushroom, time of harvest and extraction protocols might account for the variance. Likewise, anthraquinones were not detected in *T. robustus* and *T. ochracea* mushroom extracts (Table 5). Earlier works (Gbolagade and Fasidi, 2005; Wandati *et al*., 2013) also noted the absence of anthraquinones compounds in mushroom samples.

Table 5. Phytochemical constituents of mushroom extracts.

Mushroom	Saponins	Tannins	Terpenoids	Anthraquinones
L. squarrosulus	+	+	+	ND
T. robustus	+	+	+	-
T. ochraceus	+	+	+	-
X. hypoxylon	-	-	-	ND

Legend: '+' = Present; '-'= Absent; 'ND' – Not determined

4. Conclusion

In this study, 16 wild mushrooms from ligneous and terrestrial habitats were collected from ENPOST farm, Ilesa, Southwest Nigeria and the antimicrobial potential of *L. squarrosulus*, *T. robustus*, *T. ochracea* and *X. hypoxylon* were investigated. The methanolic crude extracts of the mushrooms were active against at least one of the eight bacteria, and the yeast except for *X. hypoxylon*. Generally, the extracts were active against the test isolates: *T. ochracea* > *T. robustus* > *X. hypoxylon* > *L. squarrosulus*. Among the investigated isolates, *P. aeruginosa* exhibited the highest inhibition zone (20 mm) whereas *S. aureus* had the lowest (7.2 mm). Furthermore, all the extract combinations were resistant to *K. pneumoniae* and *S. typhimurium*, all the Gram-positive bacteria and the fungus, *C. albicans*. The MIC range of 2.09 and 16.75 mg ml^{-1} was equally obtained. Also, the extracts except *X. hypoxylon* contained saponins, terpenoids and tannins. Our study reveals the antimicrobial potential of *L. squarrosulus*, *T. robustus*, *T. ochracea* and *X. hypoxylon*. However, this study is limited by the application of thorough-put techniques such as gas chromatography – mass spectrometry and fourier-transform infrared spectrometry for determination of concise constituents of extracts and time-kill assay to assess the *in-vitro* reduction of test organisms after exposure to the extracts. Extensive screening of more native mushrooms for biomedical potential and the domestication of the therapeutic species is advocated. Therefore, we recommend further investigations on isolation, evaluation, and identification of key constituent(s) with antimicrobial prospects and mechanisms of actions.

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Serum Levels of Interleukin 10, Interleukin 17A, and Calcitriol in Different Groups of Colorectal Cancer Patients

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Abstract

Colorectal cancer (CRC) is considered as the fourth prevalent reason of cancer deaths. Interleukin 10 (IL10) and interleukin 17A (IL-17A) act as anti-inflammatory and pro-inflammatory cytokines, respectively, both being able to exert antitumor or pro-tumor roles in cancer. Serum levels for IL-10, IL-17A, and calcitriol were measured by ELISA. The CRC patient sample (n =90) consisted of newly diagnosed, before surgery, and after surgery groups, the results of which were compared with those of healthy subjects (n=30). IL-10 levels demonstrated significantly lower (P<0.001), significantly higher (P<0.001), and insignificant differences in the three groups, respectively, as compared to the control. Serum IL-17A levels were significantly higher (P<0.001) in all patient groups compared with healthy subjects, whereas calcitriol levels were significantly lower in most patients. In conclusion,low levels of IL-10 found in CRC patients at diagnosis could be used as a diagnostic marker for the disease. However, chemo-radiotherapy caused an increase in the levels of this cytokine, which were restored to normal following surgical intervention. In addition, a contribution of serum calcitriol in changing the levels of IL-10 in CRC patients was observed. High levels of IL-17A, regardless of patient group, indicated a strong association with CRC.

Keywords: Colorectal cancer, Interleukin 10, Interleukin 17A, Calcitriol, Serum.

1. Introduction

CRC is considered as a malignant neoplasm resulting from the transformation of the epithelial cells lining the large intestine into malignant cells, where adenocarcinoma grows from the adenomatous polyps (Ahuja and Nettles, 2014).CRC accounts for around 608,000 deaths worldwide, being the fourth prevalent reason of cancer deaths, with an overall contribution of 8%. Despite remarkable development in clinical protocols, about 50% and 95%of CRC patients have still been reported to die in stages III and IV, respectively (Heitman *et al*., 2009).A number of factors are possibly associated with higher susceptibility to CRC development, including exposure to chemical and physical carcinogens (Ahuja and Nettles 2014) as well as beingpositive for rhesus factor (Alqudah *et al*., 2018).The treatment of CRC patients depends on several factors, including the stage of the disease; during stage I, approximately 95% of the patients are curable by surgical intervention, whereas this proportion declines to 65-80% in Stage II. In stages III and IV, more than one type of treatment is applied before surgical removal, such aschemo-radiotherapy (Ahuja and Nettles, 2014).

Some proteins secreted by immune cells are known to serve as biomarkers in cancer (Youssry et al., 2019). IL-10 is defined as an immunosuppressive protein that is produced in response to inflammatory adaptive and innate immune responses.IL-10 acts as a feedback regulator by preventing the production of cytokines by T cell/macrophages and inhibiting their antigen presenting capacity (Mittal and Roche, 2015).This cytokine is produced by T helper 1 (Th1), T helper 2 (Th2), and T regulatory (T-reg) cells, but also by macrophages and monocytes (Taylor*et al*., 2006). In addition, T helper 17 (Th17) cells can control immune responses negatively by secreting some immunosuppressive factors,e.g.IL-10, and hence called non-pathogenic Th17cells (Wu et al., 2018). IL-10 can suppress the response of both pro-inflammatory Th17 cytokines and macrophages by inhibiting certain inflammatory cytokines, such as IL-6 and IL-12. IL-10 deficiency leads to the stimulation of inflammatory responses, including the possibility of spontaneous tumor development in humans (Oft, 2014).

Several lines of evidence suggested the potential use ofIL-10 as a prognostic factor in CRC. High levels of IL-10 are associated with poor prognosis in CRC patients (Li

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,2019). An earlier study also reported a decreased serum IL-10 level in these patients (Abtahi, 2017).

IL-17A isanotherprotein that isproducedby Th17 cells. Previously reported evidence suggested a dual role for IL-17 in cancer. It can act as an antitumor cytokine by promoting cytotoxic T cell responses, leading to regression of the tumor. In contrast, it can encourage angiogenesis and migration of cancer cells (Murugaiyan and Saha, 2009). The anti-tumor activity of IL-17 is mediated by decreasing IL-10 and IL-13 levels, along withincreasing those of other interleukins, such as IL-6 and IL-12 (Razi *et al*., 2019).

IL-17A is produced by the epithelial cells in CRC patients, where it is inversely correlated with the expression of p53.IL-17 was also suggested as a valuable serum marker for CRC development (Radosavljevic *et al*., 2010).

Calcitriol (1α,25-dihydroxy vitamin D3) can be classified as a seco-steroid hormone. Vitamin D3 in humans is converted to calcitriol as an active final vitamin D3 metabolism product (Feldman et al., 2018). Several studies indicated that the normal range of serum calcitriol fluctuates with different stages of age, having values of 18-64, 18-78, and 25-45 pg/mL in serum samples tested from elder males, elder females, and younger adults, respectively (Reynolds*et al*., 2016; Weil *et al*., 2018). The endocrine system for calcitriol was first discovered in animals, where the decreased calcium level in serum was demonstrated to cause an increased calcitriol production(Feldman et al., 2018).Calcitriol relationship with CRC has been investigated but with controvery in the reported results.For example, an earlier study found that calcitriol level in serum decreases as the CRC stages progress (Niv et al., 1999),while another report showed no association between calcitriol and CRC development (Lee et al., 2011). Calcitriol affects certain inflammatory processes that are known to be responsible for the development of cancer, including the expression of IL1β, IL6, and IL17 cytokines, as well as the activity of cyclooxygenase 2 enzyme (Van Harten-Gerritsen *et al*., 2015). Also, calcitriol can enhance the expression of IL-10 by T cells (Heine et al., 2008).

Overall, the previously published data about the possible associations between CRC development and the levels of IL-10, IL-17A, or calcitriol are either controversial or insufficient. Therefore,conducting this study was necessaryto clarify the relationship between the changes in serum IL-10, IL-17A, and calcitriol levels and status of CRC patientsin terms of chemo-radiotherapy and surgical intervention. It is also equally essential to seek the possibility of using these parameters as diagnostic markers for CRC development and to find the potentialinterrelationship among these chemical mediators.

2. Materials and Methods

The present study included 90 patients with primary colorectal adenocarcinoma of various disease and treatment stages (44females and 46 males, age range 28-62 years), along with30 healthy subjects (14females and 16males, age range 27-63 years). Tumor lymph node metastasis-based classification was applied for the staging of the patients. Three groups of equal numbers of CRC patients (n=30) were involved in this study. First, the newly diagnosed group of patients who were not subjected to treatments and surgical intervention, involving all disease stages. Second, the before surgery group of patients who were subjected to chemo-radiotherapy, involving stages III, and IV. Third, the after surgery group of patients who were not subjected to any type of cancer treatment before and after the surgery, involving stages I and II. Patients with kidney failure, heart disease, diabetes, family history, chronic digestive problems, intestinal polyposis, smoking habit, and alcoholism were excluded from the study. Patients were diagnosed by medical consultant doctors. Permissions were obtained from the medical city hospitals in Baghdad, Iraq, and approved by the University of Technology's institutional ethical committee, Baghdad, Iraq (Ref. No. AS 1811-12-9- 2018) in accordance with the Helsinki Declaration of 1975 revised in 2000. All participants were informed about the study design and objectives and signed an informed consent before the collection of any data or samples. Table 1 lists the disease-related characteristics of the study participants.

Table 1. Disease characteristics of CRC patients involved in the present study, with number of cases based on stage, grade, and tumor location.

TNM stage	Case (n)	Tumor location	Case (n)
Stage I	23	Cecum	4
Stage II	23	Ascending colon	11
Stage III	22	Hepatic flexure	5
Stage IV	22	Transverse colon	8
		Splenic flexure	6
Grade	Case (n)	Descending colon	15
Grade1	20	Sigmoid colon	12
Grade 2	58	Recto-sigmoid junction	10
Grade3	12	Rectum	19

Blood specimens were collected from CRC patients and healthy subjects then separated by centrifugation. Serum samples were stored at -40º C until analysis. Sandwich Enzyme linked immunosorbent assay (ELISA) kits were used to measure the serum levels of calcitriol (MyBioSource-U.S.A), IL-10, and IL- 17A (Elabscience-U.S.A). The method is based on the concept of the binding of the antigen (e.g., proteins or hormones) to its specific antibody, which allows its detection in a fluid sample with very small quantities (Gan and Patel, 2013).

3. Statistical Analysis

Data were analyzed using the software package IBM SPSS Statistics version 24 for Windows 10. Parameterswere normally distributed and the results were shown as mean ±SD. Differences between the groups were tested by means of one-way ANOVA test and independent t-test. Correlations were analyzed by Pearson's correlation coefficient. The receiving operating characteristic (ROC) curve was used to find cutoff values and the area under the curve (AUC). Data were considered significant at $P < 0.05$.

4. Results

All CRC patients were divided into groups depending on sex (female and male) and age (≤50 and >50 years).

Serum levels of IL10, IL17A, and calcitriol showed no significant differences for sex and age when these groups were compared to each other .The results demonstrated that serum levels of IL-10, IL17A, and calcitriol were significantly higher (P <0.001) in the before surgery group as compared to the newly diagnosed group, as shown in Table 2.

Table 2: Serum levels of IL-10, IL-17A and Calcitriol in CRC patients in terms of sex and age for all patient (n=90) and in terms of exposure to therapy (Chemo-radiotherapy) for 60 patients without tumor removal.

Group	Case (n)	IL-10 (Mean±SD) pg/mL	*p value*	IL-17A (Mean±SD) pg/mL	*p value*	Calcitriol (Mean±SD) pg/mL	*p value*
Sex							
Female	44	18.02±3.35	>0.05	77.18±11.55	>0.05	56.21±7.66	>0.05
Male	46	19.62±2.22		85.09±10.34		52.79±6.09	
Age							
≤50	47	17.90±3.77	>0.05	82.07±3.12	>0.05	55.33±5.30	>0.05
>50	43	19.76±3.87		80.19±2.24		53.69±4.55	
Therapy*							
Without	30	11.02±2.98	<0.001	71.61±12.15	<0.001	48.85±5.57	<0.001
With	30	28.12±3.70		85.14±9.87		55.99±4.98	

* Only 60 CRC patients: Newly diagnosed group (n=30, no chemo-radiotherapy), before surgery group (n=30, neoadjuvant chemo-radiotherapy).

The results also showed that IL-10 levels were significantly lower in the serum of the newly diagnosed group (11.02±2.98 pg/mL, *P*<0.001), but significantly higher in the before surgery group (28.12±3.70 pg/mL, *P*<0.001) as compared to healthy subjects (17.41±3.20 pg/mL). However, no significant difference was observed between the after surgery group (17.33±3.37 pg/mL) and healthy subjects, as shown in Figure 1-A)

The results also revealed that serum IL-17A levels in the newly diagnosed,before surgery, and after surgery groups were significantly higher (71.61±12.15, 85.14±9.87, and 86.67±6.84 pg/mL, respectively) as compared to those in healthy subjects (27.77±5.35 pg/mL, *P* <0.001), as shown in Figure 1-B.

Calcitriol levels in the sera of the newly diagnosed and before surgery groups (48.85±5.57 and 55.99±4.98 pg/mL, respectively) were significantly lower compared to those in the healthy subjects (60.04 ±3.10 pg/mL; *P*<0.001 and *P* <0.05, respectively). No significant difference was found between healthy subjects and after surgery group (60.04 ±4.87 pg/mL vs 58.66 ±5.91 pg/mL, *P*>0.05), as shown in Figure 1-C.

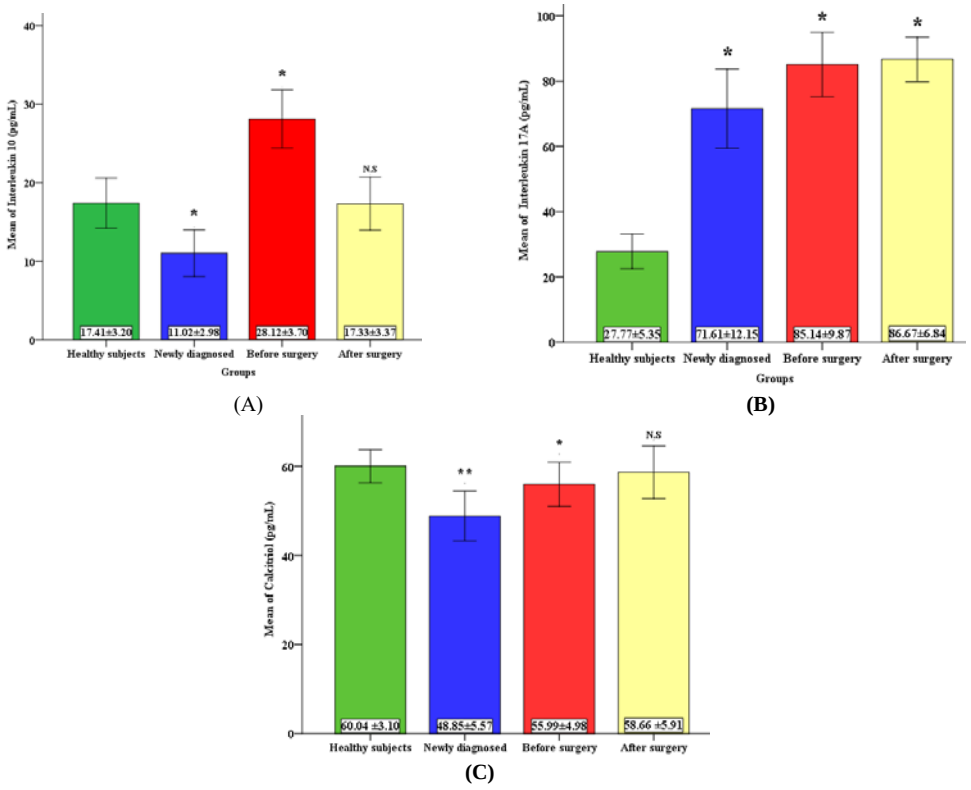
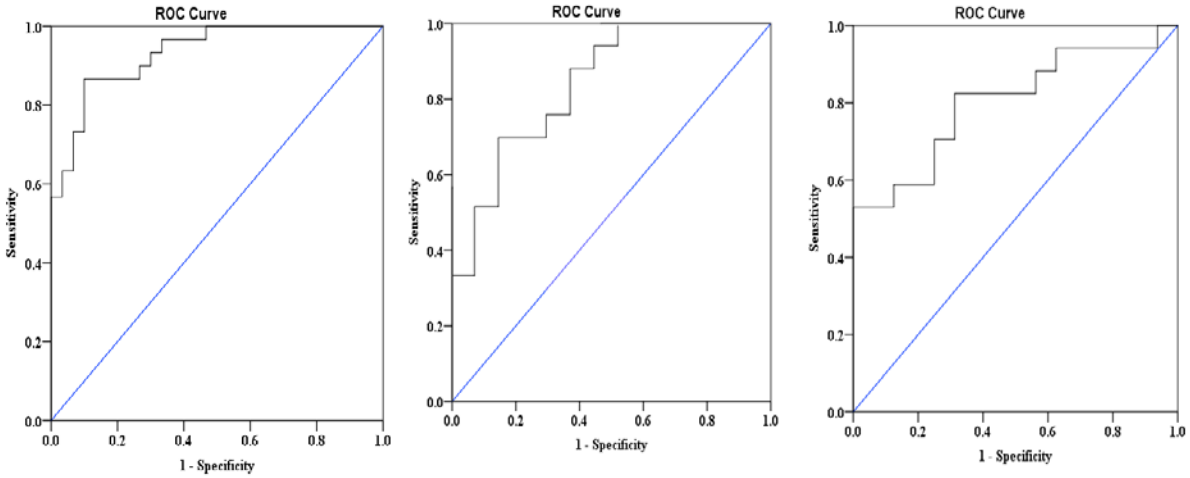


Figure 1: Levels of IL-10, IL-17A, and calcitriol in serum samples of healthy subject and CRC patient groups;**(A)**IL-10 levels in the newly diagnosed group was significantly lower while in the before surgery group was significantly higher as compared to healthy subject. No significant difference was found between the after surgery and healthy subject groups. * *P*< 0.001, N.S: Non-significant. **(B)**IL-17A levels in all CRC patient groups.The level was significantly higher as compared to healthy subjects. * *P*< 0.001.**(C)**Calcitriol levels in the newly diagnosed group and before surgery group was significant lower as compared to healthy subject. No significant difference was found between after surgery group and healthy subjects. * *P*< 0.05, ** *P*< 0.001, N.S: Non-significant.

Analysis of the ROC curve was used for testing the possibility of using IL-10, IL-17A, and calcitriol levels in the serum to diagnose CRC disease in the newly diagnosed patient group. The results showed that, for IL-10, AUC value was 0.93, the 95%-confidence interval (CI) was from 0.87 to 0.99 at *P*<0.001, and Cutoff value was 14.39 pg/mL, calculated at maximum sensitivity and specificity (87% and 90%, respectively). For IL-17A, the results showed an AUC value of 0.82, CI of0.67 to 0.97 at *P* <0.001, and Cutoff value of 67.18 pg/mL, calculated at maximum sensitivity and specificity (68% and 85%, respectively). Regarding calcitriol, the result showed an AUC of 0.80, CI of 0.64 to 0.98 at *P*<0.001, and Cutoff value of 53.14 pg/mL, calculated at maximum sensitivity and specificity (82% and 69.7% respectively), as shown in Figure 2.



(A) (B) (C)

Figure 2: ROC curve for IL-10 (A) , IL17A (B), and calcitriol (C) ; low levels of IL-10 and acalcitriol , also high levels of IL-17A, can give an indication for the presence of CRC and hence they can be used as diagnostic markers.Only one significant positive correlation was found, which was between serum levels of IL-10 and calcitriol (r =0.71; *P*<0.001),as shown in Figure 3, whereas correlations between levels of IL-17A and both IL-10 and calcitriol were non-significant, for all CRC patient groups. In the healthy subjects, all correlations between these parameters were non-significant. As shown in (Table 3),levels of IL-10, calcitriol and IL-17A showed non-significant correlations with the values of body mass index (BMI)of healthy subjects (18.6-24.2 kg/m^2) and patients (18.3-24.8 kg/m^2).In addition, significant correlations between the serum levels of these chemical mediators and age were absent, as shown in Table 4.

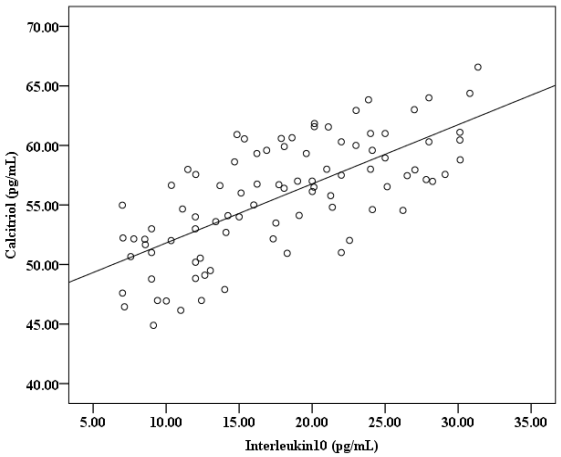


Figure 3: Significantly positive correlation between IL-10 and calcitriol in the serumof CRC patients.

Table 3. Correlations between parameters in serum for CRC patients and Healthy subjects

Correlation between	CRC Patients r (*p* value)	Healthy subjects r (*p* value)
IL-10 and Calcitriol	0.70 (<0.001)	0.45 (>0.05)
IL-10 and IL-17A	0.41 (>0.05)	0.05 (>0.05)
IL-17A and Calcitriol	0.02 (>0.05)	-0.34 (>0.05)

Table 4. Correlations between both BMI and age with the tested parameters in sera of patients and Healthy subjects

Correlation between	CRC Patients r (*p* value)	Healthy subjects r (*p* value)
IL-10 and Age	0.28 (>0.05)	0.36 (>0.05)
IL-17A and Age	-0.05 (>0.05)	-0.33 (>0.05)
Calcitriol and Age	0.05 (>0.05)	-0.02 (>0.05)
IL-10 and BMI	0.18 (>0.05)	-0.04 (>0.05)
IL-17A and BMI	-0.03 (>0.05)	-0.27 (>0.05)
Calcitriol and BMI	0.21 (>0.05)	0.06 (>0.05)

5. Discussion

Colorectal cancer patients' groups showed no significant differences in terms of age and BMI, which excludes the effects of these two factors on the studied parameters. (Table 2, 4). Also, the levels of IL-10, IL-17A, and calcitriol measured in the sera of CRC patients in the present study confirm the results obtianed by other research groups in various populations (Abtahi, 2017, Niv et al., 1999, Di Caro et al., 2016, Karabulut et al.,2016).

Levels of IL-10 in CRC patient groups involved in this study revealed differences from those in healthy subjects (Figure 1-A). IL-10 levels were significantly lower in the newly diagnosed group. IL-10, cyclooxygenase 2 (COX-2), and Interleukin 1β (IL-1β) are known to be involved in the same pathway. IL-1β was reportedto induce COX-2

synthesis through the activation of the nuclear factor κB (NF-κB), while IL-10 was reportedto block the induction of IL-1β to activate NF-κB in epithelial cells of the intestine (Al-Ashy et al., 2006, Andersen et al., 2013). COX-2 is an enzyme that increases prostaglandin secretion, where the high expression of this enzyme is associated with CRC in humans by affecting angiogenesis (Rao et al., 2004). Hence, the low levels of IL-10 in the serum reportedly lead to an increased risk of CRC disease, by elevating COX-2 expression. IL-10 deficiency increases the number of colon tumors in CRC mouse models (Tomkovich et al., 2017). A previous study showed that IL-10 level was low in the plasma of newly diagnosed CRC patients, but with non-significant differences compared with the control (Yamaguchi et al., 2019), whereas another work demonstrated significantly lower differences (Abtahi, 2017).

The present study recorded significantly highIL-10 levels in the before surgery group, which was subjected to more than one type of treatment. IL-10 tends to perform a protective function in CRC animal models, which is mediated by regulatory T cells.IL-10 was needed to decrease the tumor burden in mice (Erdman et al., 2005).

However, no significant differences were recorded in the levels of this cytokine in the after surgery group, in which tumor is completely removed without chemo-radiotherapy or any type of treatment. In a study that described CRC patients who did not experience relapse after surgery, IL-10 levels in the serum were significantly loweras compared to their state before surgery (Di Caro et al., 2016).

IL-17 is known to have a dual role in cancer because it can act as an antitumor cytokine by promoting cytotoxic T cell responses, leading to the regression of tumor (Murugaiyan and Saha, 2009).IL-17 is considered as one of the most common cytokines that promotes angiogenesis and tumor development, where it isassociated with poor prognosis in CRC (Liu et al., 2011). It is involved in a potential mechanism of stimulating the growth of tumor due to its proangiogenic effect, through enhancing the production of vascular endothelial growth factor (VEGF) which is considered as an important proangiogenic factor in many human cancer types (Razi et al., 2019).

The results of the present study showed significantly higher levels of IL-17A in the serum for all CRC patient groups (Figure 1-B).These results are consistent with those reported by previous studies related toIL-17A levels in the plasma and serum of newly diagnosed CRC patients (Yamaguchi et al., 2019, Wang et al., 2019), as well as those desribing patients before or after surgical removal (Radosavljevic et al., 2010, Karabulut et al., 2016).In the present study, the high levels of IL-17A in the after surgery group may be due to an uncompleted treatment plan, possibly involving the prescription of adjuvant chemotherapy doses to destroy the remaining cancer cells, if found.Also,the samples were collectedfrom the patients after 21 days of surgical removal, which is a short period to restore a normal health state. In addition, there is a possibility of bacterial infection for some patients as a result of surgery (Al-Awaysheh, 2018) .

Calcitriol synthesis occurs in the intestinal cells besides its synthesis in the kidney. Both receptors of vitamin D and calcidiol 1-monooxygenase can be expressed in the normal and malignant cells of the human colon (Matusiak et al., 2005). Also, a correlation exists between calcitriol levels in the serum and those in the colonic tissues in humans (Wagner et al., 2012). Calcitriol can inhibit the proliferation of the colonic epithelial cells. Therefore, low levels of calcitriol facilitate colorectal carcinoma development and affect its biological behaviour. Levels of $25(OH)D_3$ and calcitriol were reported to have different relationships in the same set of colorectal carcinoma patients. A previous study found that $25(OH)D_3$ in the serum of patients of all disease stages were higher as compared to those of the healthy individuals, whereas the levels of calcitriol were significantly lower in patients of advance stages (Niv et al., 1999).

The findings of the present work showed that calcitriol levels in the serum were significantly lower in thenewly diagnosed and before surgery groups, while the after surgery group showed no significant differences, when compared with the healthy subjects (Figure 1-C). For the newly diagnosed group, these findings confirm those published in an earlier study of CRC patients, where calcitriol level was lower in the serum (Niv et al., 1999). Various reasons can account for lower calcitriol levels in the serum. The before surgery group in this study received chemo-radiotherapy, whereas patients who received chemotherapy were less likely to be involved in outdoor activities and therefore have less exposure to sunlight. Other factors might involve modified diet regimes as well as the reported post-chemotherapy reduction of serum vitamin D levels in CRC patients (Fakih et al., 2009). The after surgery group, which included only Stage I and stage II patients, showed no significant differences in calcitriol levelsin comparison with healthy subjects.This may be due to the complete removal of the tumor before exposure toto chemo-radiotherapy regimes. A previous study showed that calcitriol levels in the serum are correlated negatively with CRC disease stages (Niv et al., 1999).The results of the current study suggest that the observed low levels of IL-10 and calcitriol in the serum of the newly diagnosed group can give an indication for the presence of the disease, hence these chemical mediator shave a good potential to be utilized as diagnostic markers(Figure 2-A,B).However, the observed low levels of IL-10 and calcitriol, which were lower than the cutoff values (14.39 pg/mL and53.14 pg/mL, respectively), may contribute in the pro-tumorconditions andassociate with an increased risk of developing CRC disease. Previous studies could not agree on a definite range of normal calcitriol value in the serum of humans. Also, several recent studies indicated an increase in the upper limits of the normal range, which reached 78 pg/ml (Feldman et al., 2018, Reynolds et al., 2016, Weil et al., 2018). Hence,this cutoff value can be regarded as acceptable to associate with the increased risk of CRC disease.

The present study showed that the high levels of IL-17A in the serum could be used to diagnose CRC disease (Figure 2- C).The recorded cutoff value (67.18 pg/mL) can aid in predictingthe magnitude of the risk to develop CRC. Previous studies also suggested that IL-17A level in the serum is an important diagnostic and prognostic marker for CRC disease (Radosavljevic et al., 2010, Wang et al., 2019).

This study also demonstrated a positive correlation between IL-10 and calcitriol (Figure 3, Table 3). A previous study showed that calcitriol could increase IL-10

expression by B cells in humans. B lymphocytes can produce the bioactive form of vitamin D (calcitriol) which enhances their expression of IL-10 Heine et al., 2008). In a study that examined the effects of calcitriol treatment on Th2 cell development in mice using an *in vitro* culture, it was found that IL-10 production was increased (Boonstra et al., 2001).

In the present study, IL-10, IL-17A, and calcitriol levels in the serum ofCRC patients who were subjected to treatment (Before surgery group) were significantly higheras compared to untreated patients(Newly diagnosed group) (Table 2, Figure1). In breast cancer patients under neoadjuvant therapy, serum levels of IL-10 were significantly increased as compared to prior use of IL-10 (Jabeen et al., 2018). IL-10 showed some resistance to chemotherapy via autocrine IL-10 production from the tumor cells in breast cancer (Yang et al., 2015). In a previous study, IL-17A was used as a biomarker of the response to bevacizumab in CRC metastases cases, where the higher levels in serum were reported to lead to shorter progression-free survival (Lereclus et al., 2017). Resistance to cisplatin drug in CRC patients was also reported (Sui et al., 2019). The high calcitriol levelsin the before surgery group may be due to vitamin D supplements that are given to CRC patient as a part of the treatment plan.

6. Conclusion

Low levels of IL- 10 in the serum can be used as a diagnostic biomarker for CRC, whereas high levels of IL-10 result from the effects of chemo-radiotherapy. The association of calcitriol with IL-10 indicates that calcitriol contributes in changing the levels of IL-10 in CRC patients. High levels of IL-17A in the serum, regardless of CRC group type, indicates a strong association with the disease. Early surgical treatment in the early stages of CRC restores the normal levels of IL-10, and calcitriol. The cutoff values found by this study can be used to predict health conditions in which the individuals are at risk or already having CRC.

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Medicinal Poisonous Plants of the Tomsk Region (Siberia)

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Abstract

In this study, we presented new data on medicinal poisonous plants of the Tomsk region, used to treat various diseases in scientific and traditional medicine. As a result of the analysis of the distribution of wild poisonous medicinal plants on the territory of the Tomsk region, a list of species used for medical treatment has been formed. Based on the analysis of geobotanical descriptions, the occurrence and abundance of most of the flora species of the Tomsk region were studied, which is the basis for a quantitative assessment of their abundance and some other indicators. The medicinal poisonous species were collected based on previous literature sources and the authors' findings in Siberia. The systematic position of each species stated – the biologically active substances (BAS) that contain the use in medicine, the distribution inside Tomsk region, and the used tissue-part.The list includes 30 species of medicinal poisonous plants belonging to 24 genera and 11 families. The richest in the composition of medicinal poisonous plants used for treating various diseases are the following families: Ranunculaceae, Boraginaceae, Solanaceae, and Apiaceae. Out of 30 species of poisonous medicinal plants, 25 species are used in folk medicine, and 5 species belong to official medicinal plants. Medicinal plants can be an alternative to chemicals and become a source of raw materials for the pharmaceutical industry.

KeyWords: Siberian Botanical Garden;Poisonous plants;Pharmacopoeia species, Folk medicine.

1. Introduction

1.1. 1.1 History of the study of medicinal plants in Siberia

Nowadays, the potential of herbal medicine for the prevention and treatment of various diseases is increasingly expanding. Medicinal plants are efficient for various treatments, including cardiovascular, nervous, and cancer diseases. Clinical trials are introduced to medicinal plants into pharmacological practice. In this regard, it is of scientific interest to study their distribution on the territory of the Tomsk region, ecological and coenotic attribution, frequency of occurrence, distribution in the administrative regions of the Tomsk region, the content of biologically active substances (BAS), and practical value. The study of medicinal plants in Siberia was first initiated by Krylov at the beginning of the last century (since 1885) in the Siberian Botanical garden (Sviridova and Kuznetsova, 2012). The scientist gardener conducts botanical excursions and collects herbarium in the vicinity of Tomsk. He participated in collection missions for the Herbarium of Tomsk State University and published the summary "Flora of Altai and Tomsk province" (1903-1914).

The summary contains keys for defining species, describing morphological features and their ecological and coenotic attribution. The final publication of Flora of Western Siberia was completed in 1964. In the future, the study of the flora of the Tomsk region continued by researchers Prokopiev, Vyltsan, Lvov, etc. In 1985, botanists Polozhiy, Revushkin, and Baranova prepared the textbook "Determinant of plants of the South of the Tomsk region". In 1994, Vyltsan completed the creation of the first "Determinant of plants of the Tomsk region" (Vyltsan, 1994). The study provides information about 920 species of vascular plants from 406 genera and 99 families. Many findings of the Tomsk region were by Ebel. In 2014, a team of authors published the "Determinant of plants of the Tomsk region" (Ebel et al., 2014). For each species, this publication provides a number of characteristics: flowering time, ecological and coenotic attribution, frequency of occurrence, distribution in the administrative regions of the Tomsk region, and practical value.

1.2. 1.2 History and priority areas of the Siberian Botanical Garden

In the 1970s, the Siberian Botanical Garden of Tomsk State University collected new 500 medicinal plants under Revina. Sviridova, Zinner and co-authors found that introduced plants in the South of the Tomsk region in a number of economically valuable features are not inferior to wild species (Sviridova and Kuznetsova, 2012; Zinner, 2010; Zinner, 2011). Currently, botanists of the Siberian Botanical garden of Tomsk State University are engaged in issues of reproductive biology and search for prospective species of medicinal plants (Belyaeva et al., 2014; Kharina and Pulkina, 2016**;**Nekratova, 2016). The priority direction is the study of medicinal plants used for the treatment of socially significant diseases, including nootropic, antitumor, hemorheological, anti-ulcer effects in natural habitats and their introduction (Kharina and Pulkina, 2016;

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Nekratova, 2016; Revina et al., 1983; Sviridova and Zibareva, 1989;Krylova et al.,2014; Zinner, 2011).

1.3. 1.3 Medicinal plants of the Tomsk region

In the work "Decorative herbaceous plants of Siberia in culture" Amelchenko, Revyakina (2007) provide information about 42 medicinal plants of the Tomsk region, among which predominate representatives of the *Ranunculaceae* families: *Adonis apennina* L., *Aconitum barbatum* Patrin ex Pers., *Thalictrum foetidum* L., *Anemone altaica* Fisch. exC.A.Mey., *Anemone sylvestris* L., *Anemone patens* L., *Alfredia cernua* (L.) Cass., *Ligularia glauca* (L.) O.Hoffm., *Artemisia gmelinii* Web. ex Stechm., *Artemisia dracunculus* L., *Fragaria moschata* Duch., *Filipendula vulgaris* Moench., *Sibbaldianthe bifurca* (L.) Kurtto & T.Erikss., *Potentilla erecta* (L.) Raeusch.

1.4. 1.4 Rare plants of the Siberian Botanical Garden

According to the data given in the monograph "Rare plants of the natural flora of Siberia ..." (2015) (Prokopiev et al., 2015), the Siberian Botanical garden has 289 species of rare plants of the Siberian flora belonging to 166 genera and 65 families. Of these, 74 species belong to medicinal plants growing in the Tomsk region, including *Athyrium filix-femina* (L.) Roth, *Matteuccia struthiopteris* (L.)Tod., *Aconitum volubile* Pallas ex Koelle, *Adonis apennina* L., *Adonis vernalis* L., *Anemone altaica* Fischer ex C.A. Meyer, *Anemone flavescens* Zucc.,*Thalictrum foetidum* L., *Trollius asiaticus* L., *Paeonia anomala* L., *Dianthus deltoides* L., *Dianthus superbus* L., etc.

1.5. Flora of the Tomsk region

The Tomsk region is still pointedly needed for more flora studies other Siberia regions because of the remote areas. According to the data provided in "Determinant of plants of the Tomsk region" (Ebel et al., 2014) vascular plants of the Tomsk region represented 1170 species belonging to 477 genera and 111 families. The spectrum of leading families is as follows: *Compositae*(142 species), *Poaceae* (113), *Cyperaceae* (72), *Brassicaceae* (61), *Rosaceae* (59), *Leguminosae*(54), *Ranunculaceae* (47), *Caryophyllaceae* (46), *Scrophulariaceae* (36), *Polygonaceae* (35), *Lamiaceae* (32), *Apiaceae* (29).

1.6. Red Book of the Tomsk region

The Red Data Book of the Tomsk Region (2013) includes 36 species of rare medicinal plants of Siberia (higher spore-bearing plants, gymnosperms and angiosperms: *Botrychium multifidum* (S.G.Gmelin) Rupr., *Dryopteris filix-mas* (L.) Schott, *Juniperus communis* L., *Aconitum anthora* L. ,*Actaea spicata* L., *Silene viscosa* (L.) Pers., *Persicaria vivipara* (L.) Ronse. Decr., *Hypericum ascyron* L., *Primula veris* subsp. *macrocalyx* (Bunge) Ludi, *Tilia cordata* Miller , etc.

Here is a map of the research area (Tomsk region) (Figure 1).

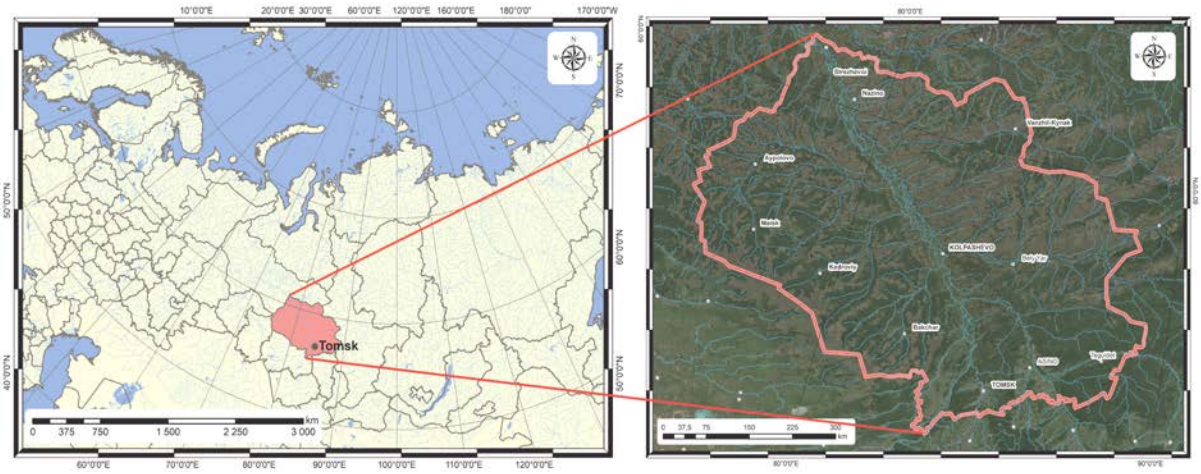

Figure 1 Map of the Tomsk Region

2. Materials and Methods

The medicinal poisonous species were collected based on previous literature sources (Ebel et al., 2014; Amelchenko, 2010; Amelchenko and Revyakina, 2007) and the authors' findings in Siberia (Nekratova and Shilova, 2015; Nekratova, 2015; Nekratova and Shilova, 2016; Nekratova and Letchamo, 2018; Nekratova and Kosmodemyanskiy, 2019). Based on the analysis of geobotanical descriptions, the occurrence and abundance of most of the flora species of the Tomsk region were studied, which is the basis for a quantitative assessment of their abundance and some other indicators. The listed indicators are mainly among studies of the plant ecology and their resources, which constitute the scientific basis for the rational use of flora and vegetation of any region. In addition, the occurrence of species and their abundance made it possible to scientifically substantiate the identification of rare species in the flora of the Tomsk region, which is an initial and very important stage for organizing their practical protection.

3. Results and Discussion

As a result of the analysis of the distribution of wild medicinal poisonous plants on the territory of the Tomsk region, a list of species used for treatment in both folk and scientific medicine was formed (Table 1). To compile the list of species, the following literary sources were used

(Amelchenko, 2010; Ebel, 2014; Minaeva, 1991; Nekratova, 2015; Prokopiev et al., 2015; Shilova et al., 2010; State Pharmacopoeia …, 2018; Wild useful plants of Russia, 2001 etc.). To compile the list of species, 42 literary sources were used. All sources are listed in the bibliography.

The list includes 30 species of medicinal poisonous plants belonging to 24 genera and 11 families. The richest in the composition of medicinal plants used for the treatment of various diseases are the following families: *Ranunculaceae* (13 species), *Boraginaceae* (3 species), *Apiaceae* (2 species), Asparagaceae (2 species), Nymphaeaceae (2 species). Out of 30 species of medicinal poisonous plants, 25 species are used in folk medicine, 5 species belong to official medicinal plants (Table 1).

About some species used the aerial tissues in the medicine, others used the underground tissues, and some used particular organs. Of the entire list of medicinal plants, 17 species could classify as frequently occurring, common 1 species, rare 9 species, and the remaining species are infrequent and rare (Ebel et al., 2014).

The BAS content of Tomsk medicinal poisonous plants showed 16 species contain flavonoids: *Adonis apennina* L., *Anemone patens* L.,*Chelidoniu mmajus* L., *Cicuta virosa* L., *Clematis alpina* subsp. *sibirica* (L.) Kuntze, *Conium maculatum* L. , *Convallaria majalis* L., etc.

Tannins that are polyphenol complex with flavonoids found in 12 species of medicinal poisonous plants of the Tomsk region: *Actaea cimicifuga* L., *Cynoglossum officinale* L., *Nuphar lutea* (L.) Smith., *Nymphaea candida* C., *Symphytum officinale* L., *Thalictrum flavum* L., *Thalictrum foetidum* L., etc.

Most species of medicinal poisonous plants are alkaloid-containing (26 species): *Aconitum anthora* L, and other species of this genus, *Actaea cimicifuga* L., *Adonis apennina* L., *Chelidonium majus* L., *Cicuta virosa* L., *Conium maculatum* L., *Cynoglossum officinale* L.,*Datura stramonium* L.,etc.

Six medicinal poisonous species having essential oils are *Chelidonium majus* L., *Cicuta virosa* L., *Conium maculatum* L., *Convallaria majalis* L., *Nymphaea candida* C. Presl , *Paeonia anomala* L.

Seven species vitamin-bearing are *Adonis apennina* L., *Chelidonium majus* L., *Clematis alpina* subsp. *sibirica* (L.) Kuntze, *Conium maculatum* L., *Echium vulgare* L., *Polygonatum odoratum* (Mill.) Druce, *Xanthium strumarium* L.

Fourteen medicinal poisonous species, including saponins are *Actaea cimicifuga* L., *Clematis alpina* subsp. *sibirica* (L.) Kuntze, *Echium vulgare* L., *Paris quadrifolia* L., *Paeonia anomala* L., *Polygonatum odoratum* (Mill.) Druce, *Scrophularia nodosa* L., etc.

Three medicinal poisonous species containing cardiac glycosides are: *Adonis apennina* L., *Clematis alpina* subsp. *sibirica* (L.) Kuntze,*Convallaria majalis*L.

However, there are very few species synthesizing ecdysteroids in the flora of poisonous medicinal plants of the Tomsk region: *Paris quadrifolia* L.

Table 1 - List of poisonous plants of the Tomsk region used in the treatment of diseases

No.	Species/Family	BAS	Application in medicine	Status in Tomskregion	Tissues used
1.	*Aconitum anthora* L. **Ranunculaceae**	Alkaloids, phytosterols, fatty acids (Wild useful, 2001)	In folk medicine(Amelchenko, Revyakina, 2007; Prokopiev *et al.,*2015). Antitumor agent. Nervous diseases (anticonvulsant, sedative) (Minaeva, 1991). Rheumatism. Tincture 100 g / 1 liter of alcohol, 1 tablespoon each for external use (Balakirev *et al*., 2006).	Very rarely (Ebel *et al*., 2014)	Underground part
2.	*Aconitum barbatum* Patrin. ex Pers. **Ranunculaceae**	Alkaloids delasin, lycoctonine, tangorin, batocanin (Minaeva, 1991)	In folk medicine. Antitumor agent. Nervous diseases (anticonvulsant, sedative) (Minaeva, 1991). Rheumatism. Tincture 100 g / 1 liter of alcohol, 1 tablespoon each for external use (Balakirev *et al*., 2006).	Rarely (Ebel*et al*., 2014).	Underground part
3.	*Aconitumseptentrionale* Koelle **Ranunculaceae**	Alkaloids, phytosterols, fatty acids (Wilduseful ..., 2001; Lapin *et al*., 2004).	In folk medicine. Antitumor agent. Nervous diseases (anticonvulsant, sedative) (Minaeva, 1991). Rheumatism. Tincture 100 g / 1 liter of alcohol, 1 tablespoon each for external use (Balakirev *et al*., 2006).	Occasionally (Ebel*et al.*, 2014).	Underground part
4.	*Aconitum volubile* Pallas ex Koelle **Ranunculaceae**	Alkaloids, phytosterols, fatty acids(Minaeva, 1991)	In folk medicine. Antitumor agent. Nervous diseases (anticonvulsant, sedative) (Minaeva, 1991). Rheumatism. Tincture 100 g / 1 liter of alcohol, 1 tablespoon each for external use (Balakirev *et al*., 2006).	Occasionally (Ebel*et al*., 2014).	Underground part

Cont. Table 1

5.	*Actaea cimicifuga* L. **Ranunculaceae**	Triterpenoids, tannins, saponins, alkaloids, resins, phytosterols, salicylic and isoferulic acids (Minaeva, 1991; Wilduseful ..., 2001).	In folk medicine. Cardiovascular diseases (hypotensive, anti-sclerotic) (Minaeva, 1991). Antitumor agent (leukemia) (Wilduseful ..., 2001). Tincture 2/10 on 70% alcohol (Pashinsky, 1989)	Often (Ebel*et al.,* 2014).	Rhizomes withroots
6.	*Adonisapennina*L. **Ranunculaceae**	Cardiac glycosides, alkaloids, flavonoids, and vitamin C (Minaeva, 1991).	In folk medicine (Amelchenko andRevyakina, 2007; Prokopiev*et al.,*2015). Cardiovascular diseases. Nervous diseases (sedative) (Minaeva, 1991). Infusion of 1-2 teaspoons of dry raw materials for 1 glass of boiling water, take 1 tablespoon 3 times a day) (Balakirev *et al*., 2006).	Rarely (Ebel*etal.*, 2014).	Aerialpart
7	*Anemone patens* L. **Ranunculaceae**	Anemonin, triterpenecompounds, flavonoids, phytoncides, phytosterols (Wild useful ..., 2001; Ulanova 1985).	In folk medicine(Amelchenko and Revyakina, 2007; Prokopiev*et al.,*2015). Nervous diseases (asthenia, epilepsy). Cardiovascular diseases (hypertension). Antitumor agent (Minaeva, 1991; Wild useful ..., 2001). Infusion of 2 teaspoons of herbs in 1 glass of boiled water, drink in equal shares throughout the day (Balakirev *et al*., 2006).	Often (Ebel*et al.,* 2014).	Aerialpart
8.	*Caltha palustris* L. **Ranunculaceae**	Protoanemonin, choline, berberine, carotene, triterpene glycosides (Minaeva, 1991).	Infolkmedicine. Antitumor effect. Nervous diseases (Minaeva, 1991). Infusion of 1/2 spoon of dry raw materials in 250 ml of hot water, take 50 ml 3 times a day before meals (Balakirev *et al*., 2006).	Often (Ebel *et al.,* 2014).	
9.	*Chelidonium majus* L. **Papaveraceae**	Alkaloids, saponins, bitter and resinous substances, flavonoids, choline, histamine, methylamine, organic acids, essential oil, carotene, vitamin C (Minaeva, 1991).	Pharmacopoeia species (State Pharmacopoeia..., 2018;Shikov *et al.,* 2021). Antitumor agent (external and internal tumors). Cardiovascular diseases (hypertension, angina). Nervous diseases (epilepsy) (Minaeva, 1991) Tincture (1/10) for 40% alcohol (Pashinsky, 1989). Infusion of 1 tablespoon of dry raw materials for 1 glass of boiling water, take 2 tablespoons 3 times a day before meals (Balakirev *et al*., 2006).	Often (Ebel *et al.*, 2014).	Aerialpart
10.	*Cicuta virosa* L. **Apiaceae**	Cycutotoxin, alkaloids, polyacetylenecompounds, coumarins, essentialoil, flavonoids (Minaeva, 1991; Wilduseful..., 2001).	In folk medicine. Nervous diseases (epilepsy, hysteria, neurasthenia) (Minaeva, 1991; Wild useful..., 2001). One of the most poisonous plants in the Russian flora. Independent use is not recommended, only use in doses selected by the doctor (Balakirev *et al.,* 2006).	Often (Ebel*etal.*, 2014).	Rhizomes withroots
11.	*Clematis alpina* subsp. *sibirica* (L.) Kuntze **Ranunculaceae**	Triterpenesaponins, protoanemonin, polysaccharides, flavonoids, caffeic and quinicacids, sugars, alkaloids, cardiac glycosides, vitamin C, microelements (Sdobnikova, Kovalevich, 1975; Kosichenko and Kozakova, 1980; Bokova *et al.*, 1982).	In folk medicine. (Prokopiev*et al.,*2015). Antitumor agent. Cardiovascular diseases: nootropic effect (Shilova et *al*., 2010); heart weakness (Minaeva, 1991). Infusion of 1 teaspoon of dry raw materials for 2 cups of boiling water, drink 1 tablespoon 3-4 times a day (Balakirev *et al*., 2006).	Often (Ebel *et al.*, 2014).	Aerialpart

Cont. Table 1

12.	*Conium maculatum* L. **Apiaceae**	Alkaloids, tannins, essential and fatty oils, flavonoids, vitamin C, carotene (Minaeva, 1991; Bulgakov *et al.*, 2016).	In folk medicine. Antitumor agent Nervous diseases (epilepsy, anticonvulsant) (Minaeva, 1991). Tincture of 50 grams of fresh flowers / 0.5 liters of alcohol (Balakirev *et al.,* 2006). Pharmacopoeia species (State Pharmacopoeia..., 2018; Shikov *et al.*, 2021)	Often (Ebel *et al.*, 2014).	Aerialpart
13.	*Convallaria majalis* L. **Asparagaceae**	Cardiac glycosides, mayalin alkaloid, essential oil, choline, organic acids, sugars, starch, flavonoids (Komissarenko *et al*., 1988).	Cardiovascular diseases (acute and chronic circulatory failure, myocardiodystrophy). Nervous diseases (in folk medicine): epilepsy, paralysis (Minaeva, 1991). Infusion of 2-6 grams of flowers per 1 glass of water, drink 1 tablespoon 3 times a day (Balakirev *et al*., 2006).	Rarely (Ebel *et al.*, 2014).	Aerialpart
14.	*Cynoglossum officinale* L. **Boraginaceae**	Alkaloidcynoglossin, glycosidin, coumarins, allantoin, choline, tannins, inulin, resins, carotene, litospermicacid, cinnamicacid, andfumaricacid (Minaeva, 1991; Yunusova *et al.,* 2012).	Infolkmedicine. Antitumor agent (tumors of the female genital area). Cardiovascular diseases (hypertension). Nervous diseases (sedative effect) (Minaeva, 1991). Infusion of 1 spoonful of raw materials for 1 glass of boiling water, take 1/2 teaspoon 3 times a day (Balakirev *et al*., 2006). Pharmacopoeia species (State Pharmacopoeia..., 2018; Shikov*et al.*, 2021).	Very rarely (Ebel *et al*., 2014).	Roots
15.	*Datura stramonium* L. **Solanaceae**	Alkaloidatropine, scopolamine, carbohydrates, phenolicandfattyacids (Minaeva, 1991; Wild useful ..., 2001).	Nervous diseases (manic-depressive psychosis, epilepsy, hysteria). Antitumoragent (stomachcancer) (Minaeva, 1991; Wild useful ..., 2001). 1 drop of dope juice with 2 tablespoons of water 3 times a day only under medical supervision (Balakirev *et al*., 2006).	Very rarely (Ebel*et al.*, 2014).	Fruits
16.	*Echium vulgare* L. **Boraginaceae**	Saponins, alkaloids, fattyoil, vitaminC, choline, phenolicandfattyacids (Minaeva, 1991; Wilduseful ..., 2001; Kruglov andSvechnikova, 2007).	In folk medicine. Nervous diseases (epilepsy, convulsions, fright) (Minaeva, 1991; Wild useful..., 2001). Infusion of 10 grams of raw materials per 300 ml of boiling water, take 15 ml 3 times a day (Balakirev *et al*., 2006). Pharmacopoeia species (State Pharmacopoeia..., 2018; Shikov*et al*., 2021).	Rarely (Ebel *et al*., 2014).	Flowers and leaves
17.	*Hyoscyamus niger* L. **Solanaceae**	Atropine group alkaloids, glycosides, fatty oil, rutin flavonoid, phenolic and fatty acids, etc. (Minaeva, 1991; Wild useful..., 2001).	Nervous diseases (manic-depressive psychosis, neurosis, hysteria) (Wild useful..., 2001). Extract 0.25 grams of dry raw materials per 100 ml of boiling water. Take orally 2 drops of the extract per 1 tablespoon of water 3 times a day (Balakirev *et al.*, 2006).	Occasionally(Ebel *et al.*, 2014).	Leaves and seeds

Cont. Table 1

18.	*Nuphar lutea* (L.) Smith. **Nymphaeaceae**	Alkaloids, phytosterols, carbohydrates, tannins, phenolic and fatty acids (Wilduseful ..., 2001; Maksyutina *et al.*, 1985; Fedotcheva *et al.,* 2016).	In folk medicine. Cardiovascular diseases (hypertension). Antitumor agent (Fedotcheva *et al*., 2016). Nervous diseases (insomnia) (Minaeva, 1991; Wild useful..., 2001). Infusion of 20 grams of dry raw materials per 1 liter of boiling water, take 1 tablespoon 3 times a day (Balakirev *et al*., 2006).	Often (Ebel *et al*., 2014).	Rhizomes
19.	*Nymphaea candida* C. Presl **Nymphaeaceae**	The alkaloid nymphein, tannins, starch, glucose, essential oil, and the glucosidenymphalin (Minaeva, 1991).	In folk medicine. Nervous diseases (soothing, hypnotic effect). Cardiovasculardiseases (Minaeva, 1991). A decoction of 1 teaspoon of raw materials for 1 cup of boiling water, take 3 times a day, 1 teaspoon (Balakirev *et al*., 2006).	Often (Ebel *et al.,* 2014).	Flowers
20.	*Paris quadrifolia* L. **Melanthiaceae**	Glycosides, alkaloids, saponins, ecdysterone, polypodine(Novoselskaya *et al*., 1981).	In folk medicine. Cardiovascular diseases (heart failure, tachycardia, arrhythmia). The nervous disorder (convulsions, somnolence) (Minaeva, 1991). It is recommended to use only in dosages selected by a doctor and under the supervision of a doctor (Minaeva, 1991).	Often (Ebel *et al.,* 2014).	Aerialpart
21.	*Paeonia anomala* L. **Paeoniaceae**	Essential oil, peonoside, peonoflorin, iridoids, salicylic and gallic acids, flavonoids, saponins, sterols, tannins, sugars, starch, salicin glycoside, resins, etc. (Minaeva, 1991; Zaripova *et al*., 2005).	In folk medicine(Amelchenko andRevyakina, 2007; Prokopiev*et al.,*2015). Nervous diseases (sedative, anti-stress effects in insomnia, neurasthenia, epilepsy) (Romanova*et al.*, 2014).Cardiovascular diseases (vegetative-vasculardisorders, hypertension) (Minaeva, 1991).Antitumoragent. Alcoholic 10% root tincture, 30-40 drops are used 3 times a day (Balakirev *et al.,* 2006 ; Pashinsky, 1989).	Often (Ebel *et al.,* 2014).	Underground part
22.	*Polygonatum odoratum* (Mill.) Druce **Asparagaceae**	Alkaloids, steroids, carbohydrates, flavonoids, saponins, mucus, sugars, vitamin C(Minaeva, 1991; Wilduseful ..., 2001; Belenovskayaand Budantsev, 2014;Strigina *et al.,* 2003).	In folk medicine (Amelchenko, Revyakina, 2007; Prokopiev*et al.,*2015). Cardiovasculardiseases (Minaeva, 1991). A decoction of 40-50 grams of raw materials per 0.5 liters of water (Balakirev *et al*., 2006).	Often (Ebel *et al*., 2014).	Rhizomes
23.	*Scrophularia nodosa* L. **Scrophulariaceae**	Alkaloid scrofularin, saponins, iridoids, carbohydrates, flavonoids, coumarins, organic acids, tannins (Minaeva, 1991).	In folk medicine(Prokopiev*et al.,*2015). Antitumor agent. Nervous diseases (insomnia) (Minaeva, 1991). Infusion of 1 teaspoon of raw materials in a glass of boiling water, drink 1/3 cup during the day (Balakirev *et al*., 2006).	Usually (Ebel *et al.,*2014).	Roots

Cont. Table 1

24.	*Symphytum officinale* L. **Boraginaceae**	The alkaloid cynoglossin, glucoalkaloidconsolidated,iridoids, polysaccharides, steroid saponins, coumarinscopoletin, allantoin, tannins monoterpene glycosides, oxycoric acids (Minaeva, 1991; Lyashenko*et al.,* 2015).	In folk medicine. Nervous diseases (anticonvulsant activity). Antitumoragent (Minaeva, 1991). Infusion of 1 teaspoon of raw materials in a glass of boiling water, drink 1/3 cup during the day (Balakirev *et al*., 2006).	Very rarely (Ebel *et al.*, 2014).	Rhizomes
25.	*Thalictrum flavum*L. **Ranunculaceae**	Triterpenesaponins, flavonoids, coumarins, resins, tannins, organic acids, alkaloids (berberine, talmin, glaucin, etc.) (Minaeva, 1991).	Infolkmedicine. Cardiovascular diseases (hypertension). Antitumor agent (Minaeva, 1991). Alcoholic tincture on 40% alcohol, 15-20 drops 2-3 times a day in the afternoon (Pashinsky, 1989).	Often (Ebel *et al.,* 2014).	Aerialpart
26.	*Thalictrum foetidum* L. **Ranunculaceae**	Triterpenesaponins, flavonoids, coumarins, resins, tannins, organic acids, alkaloids (berberine, talmin, glaucin, etc.) (Minaeva, 1991;Savelieva *et al.*, 2016).	In folk medicine(Amelchenko and Revyakina, 2007; Prokopiev *et al.,*2015). Cardiovascular diseases (hypertension). Antitumor agent (sarcoma and other tumors) (Minaeva, 1991). Alcoholic tincture on 40% alcohol, 15-20 drops 2-3 times a day in the afternoon (Pashinsky, 1989).	Often (Ebel *et al.,* 2014).	Aerialpart
27.	*Thalictrum minus* L. **Ranunculaceae**	Triterpenesaponins, flavonoids, coumarins, resins, tannins, organic acids, alkaloids (berberine, talmin, glaucin, etc.) (Minaeva, 1991).	In folk medicine. Cardiovascular diseases (hypertension).Antitumor agent (Minaeva, 1991). Alcoholic tincture on 40% alcohol, 15-20 drops 2-3 times a day in the afternoon (Pashinsky, 1989).	Often (Ebel *et al.,* 2014).	Aerialpart
28.	*Thalictrum simplex*L. **Ranunculaceae**	Triterpenesaponins, flavonoids, coumarins, resins, tannins, organic acids, alkaloids (berberine, talmin, glaucin, etc.) (Minaeva, 1991).	Infolkmedicine. Cardiovascular diseases (hypertension).Antitumor agent (Minaeva, 1991). Alcoholic tincture on 40% alcohol, 15-20 drops 2-3 times a day in the afternoon (Pashinsky, 1989).	Often (Ebel *et al.,* 2014).	Aerialpart
29.	*Veratrum lobelianum* Bernh. **Melanthiaceae**	Alkaloids, glycoside veratromarin, triterpenoids, amino acids, resins, gum, fatty oil, starch, sugars, mineral salts, coloring and tannins (Minaeva, 1991).	Pharmacopoeia species (State Pharmacopoeia..., 2018; Shikov*et al.*, 2021). Cardiovascular diseases (hypertension). Nervous diseases (epilepsy, melancholy) (Minaeva, 1991). It is recommended to use only in dosages selected by a doctor and under the supervision of a doctor (Minaeva, 1991).	Often (Ebel *et al.,* 2014).	Rhizomes and roots
30.	*Xanthium strumarium* L. **Compositae**	Iodine, flavonoids, alkaloid, vitamin C, resins, saponins, terpenoids, xanthostrumarin (Minaeva, 1991; Wild useful ..., 2001, Bubenchikova *et al.,* 2009).	In folk medicine. Antitumor agent (throat tumors) (Minaeva, 1991; Korepanov*et al.*, 2011). Infusion of 2 teaspoons of raw materials for 1 cup of boiling water, take 1/2 cup 3 times a day before meals (Balakirev *et al*., 2006).	Very rarely (Ebel *et al*., 2014).	The whole plant (aerial part and roots)

4. Conclusion

In this study, we presented new data on medicinal poisonous plants of the Tomsk region, used to treat various diseases in scientific and traditional medicine. Flora of poisonous medicinal plants of the Tomsk region includes 30 species belonging to 24 genera and 11 families. The Tomsk region is rich in medicinal and poisonous plants. This list may not include all species. We are planning to expand our list. However, Tomsk pharmacologists are actively studying medicinal plants of Siberia, and in the future we plan to expand the list with new types of medicinal plants. Medicinal plants can be an alternative to chemicals and become a source of raw materials for the pharmaceutical industry.

5. Conflict of interests

The authors declare that there is no conflict of interest concerning this work or the preparation of the manuscript.

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Histopathological Analysis of Striped Catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) Spontaneously Infected with *Aeromonas hydrophila*

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Abstract

Mass mortality of *Pangasianodon hypophthalmus* in cultured earthen pond was investigated. Infected *P. hypophthalmus* were characterized by number of clinical signs such as abnormal swimming behaviour, skin lesion, and local hemorrhages. Moribund samples were collected from Karnataka to investigate the etiological as well as histopathological changes. The causative agents of the diseased pangasius were identified as *Aeromonas hydrophila* using specific selective media, polymerase chain reaction (PCR) technique and through scanning electron microscopy (SEM). The amplified DNA product was 130bp for haemolysin genes of *A. hydrophila*. Scanning electron microscopy revealed straight rods with single polar flagellum of *A. hydrophila*. Histopathogical studies of different organs of diseased *P. hypothalamus* revealed cellular alteration such as necrosis and hemorrhages in the gill and liver, tubular degenerations in the kidneys, splenitis accompanied with hemosiderosis in spleen. Additionally, there was sloughed necrotic debris in intestinal lumen with increase in goblet cell number.

Keywords, Histopathology, *Aeromonas hydrophila*, *Pangasianodon hypophthalmus*, PCR, SEM

1. Introduction

Over the last few decades, aquaculture has played a vital and steadily rising role in food safety and monetary stability to the world (Houston *et al.,* 2020). Aquatic animals derived from aquaculture became the fundamental source of high-quality protein for human consumption. World fisheries production had reached at apex in 2017, producing 172.6 million tons with aquaculture contributing approximately fifty percent and the rest from capture (FAO, 2019). The modern technology has heighted the production of economically profitable fish species such as carps and catfishes, and thus the aquaculture system transformed from extensive to intensive and super intensive system. Striped catfish, *Pangasianodon hypophthalmus* (*P. hypophthalmus*) is *an* exotic fish species introduced in India from Bangladesh in 1997. Since its entry to India, pangasius has been playing a major role in many aspects: employment generation, cash flow, protein source, utilization of derelict ponds without disturbing the environment and indigenous fish species. Due to its unique fast growth capacity, pangasius become the third most cultivable fish species after rohu and catla and is highly desirable in export market (Singh and Lakra, 2012). In domestic market, pangasius has reasonable market price and possessed highly quality protein, essential amino acids, fatty acids, vitamins, and minerals in its flesh (Rahman *et al.*, 2020). Recently, it has made its entry into ornamental fish markets in different parts of the world. The IUCN Red List has listed the pangasius catfish, *P. hypophthalmus*, of the family Siluriformes, order Pangasiidae, as an endangered freshwater fish species native to the Mekong delta (Vidthayanon and Hogan, 2013). Viet Nam has ranked top in pangasius production, generating nearly 11, 41000 tons (Nguyen, 2013). Though *P. hypophthalmus* has been regarded as highly disease resistant fish, recent report revealed that several diseases affect this fish in juvenile (Kumar *et al.,* 2018; Mamun *et al.,* 2020) as well as adult stages (Kumar *et al.,* 2013; Kumar *et al.,* 2015) causing a significant economic loss in Indian aquaculture.

Many bacterial infections threaten this industry including *Edwardsiella icataluri* (Yuasa *et al.,* 2003; Ferguson *et al.,* 2001) and *Aeromonas* spp. especially with *A. hydrophila* (Subagja *et al.,* 1999; Crumlish *et al.,* 2010; Elgendy *et al*., 2017)) in East Asia and *Enterobacter cloacae* (Kumar *et al.,* 2013) and *Aeromonas jandaei* (Kumar *et al.,* 2015) in India. However, reports were scanty on *A. hydrophila* infection in pangasius catfish from cultured ponds. Usually in pond aquaculture, the water management is not well controlled, and in dry season sometimes water level reaches below the optimum level. In turn, fish get affected by stress, and this leads to the

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severe disease outbreak. High stocking density, irregular feeding, over feeding accompanied with lack of scientific knowledge may exacerbate the disease condition in such levels where the recovery would become impossible. Proper isolation and identification of bacterial infection can provide essential knowledge to the farmers for prevention and health management. Moreover, histopathological examination of tissue biopsies for the identification of aeromonads infection is extremely important. Therefore, present study was carried out for the isolation, identification, and histopathological studies of naturally infected pangasius catfish collected from natural pond of Karnataka.

2. Materials and Methods

2.1. Bacterial isolation from diseased fish

Moribund fish samples (n=10) were collected from the cultural pond of Mudigere (Lat.13°07′19″N; Long.75°37′38″E.) to the College of Fisheries, Mangalore, Karnataka, India in packed plastic bags containing 1/3rd of habitat water and 2/3rd oxygen. Moribund fishes displayed several clinical symptoms: numerous haemorrhagic spot all over the body, surface, rectal distension, and anemic body (Fig. 1a-c). Diseased fish were euthanized, and alcohol washed in order to avoid contamination. Swab samples of lesions, body cavity, and kidney were taken and streaked on the Rimler-Shotts (RS) media for the presumptive isolation of *A. hydrophila*. The pure colonies from RS agar plates were randomly picked, cultured on 1.5% Tryptone Soya Broth (TSB) and stored as streaks in Brain Heart Infusion (BHI) agar (Himedia, Mumbai) slants.

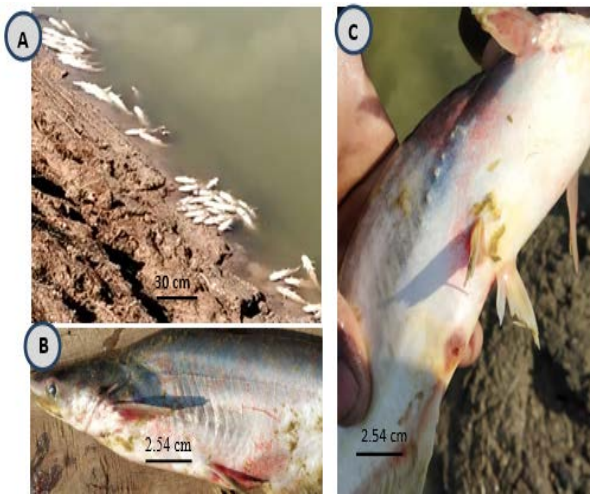

Figure 1. Mass mortality of pangasius in earthen pond of Karnataka **(A)** Diseased fish showing haemorrhages in the belly and fin bases **(B)**, rectal protrusion of *P. hypophthalmus* **(C)**

For DNA extraction, the bacterial isolates were grown on 1.5% tryoptone soya broth (Himedia) and harvested at OD 0.7 by centrifugation for 5 min at 4 °C at 5000 rpm.

Extraction of DNA was carried out by DNA-XPress™ Reagent (Himedia, Mumbai) and manufacturer's instruction were followed. Polymerase Chain Reaction (PCR) was performed in 0.2 ml micro-fuge tubes (Bio-rad, USA) in a programmable thermo cycler (C1000 Touch™ Bio-Rad, USA) using the haemolysinF: 5′-GCCGAGCGCCCAGAAGGTGAGTT-3′ and haemolysinR : 5′- GAGCGGCTGGATGCCGTTGT-3′ primers (Wang *et al.,* 2003). In PCR amplification, initial denaturation at 95°C for 5 min was maintained. Then, 39 cycles of denaturation, annealing, and extension steps were done at 95°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec respectively. The final extension was carried out for 7 min at 72°C. The amplified PCR products were mixed with 6X-gel loading dye (Genei, Bangaluru) at 10:3 ratios and loaded onto 1.5% agarose gel and the product was electrophoresed at 130 V for 30 min. The amplicon was observed under U.V transilluminator (Major Science SmartView Pro Imager UVCI-2300, USA) and documented.

2.2. Field Emission Scanning Electron Microscopy

Bacterial species were characterized in FE-SEM according to the manufacturer's instruction. A small quantity of prepared bacterial broth was dispersed on a SEM specimen mount. Enough argon was maintained in the chamber so that the vacuum reads 0.08 mbar. Sample was coated with gold (Eiko 1B-3 at 0.15 torr) as appropriate for a specified period of time and current to obtain an acceptable coating. Finally, the sample was transferred back to the specimen box after coating for analyzing at Carl Zeiss Sigma VP Field Emission Scanning Electron Microscope in the Central Laboratory of DST-PURSE PROGRAMME, Mangalore University.

2.3. Histopathology of diseased P. hypophthalmus

For histopathological study, moribund fish were euthanized, and different organs such as gills, liver, kidney, spleen and intestine were cut-off, washed with physiological saline and fixed in 10% buffered formalin for 72 h. For each sample, 3 fish were taken. For intestine, up to 10 villi per slide and no less than five were considered in the present study. Following the fixation, dehydration was done in series of alcoholic solutions in lower to higher concentration. Tissue was embedded with paraffin wax and sectioned at 5-6 µm (Thermo Scientific, Microm HM-325, USA). The counter stains, haematoxylin and eosin, were used in this study. All the histological procedures were followed as detailed by Bullock (1989). The tissues sections were photographed with the light microscope (Olympus, BX3-25ND25, Japan).

2.4. Challenge with A. hydrophila

Isolated *A. hydrophila* were revived from -40 ºC and grown on to 1.5% TSB. The bacterial broth was harvested at OD 0.7 to achieve 10^7 CFU/ml. Juvenile pangasius catfish weighing 20.5 ±5.35 were used for challenge studies. Each juvenile fish (n=10) was challenged (intramuscular) with 0.2ml of bacterial broth (*A. hydrophila*) and for control (n=10) 0.2 ml PBS were injected. Challenged fish were monitored for 10 days to check the clinical symptoms. Fish were sacrificed prior to the approval of the animal ethics committee, College of Fisheries, Mangalore.

3. Results

3.1. Presumptive and molecular identification of A. hydrophila

Yellow colonies were grown on the RS medium. The DNA amplified product of the desired size was 130bp (Fig. 2).

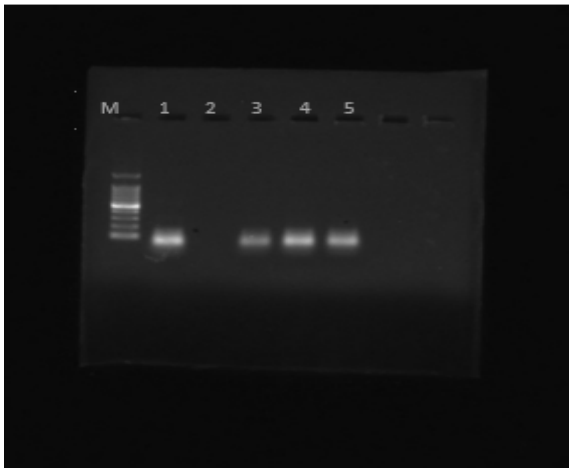

Figure 2. Detection of *Aeromonas hydrophila* by PCR. Lane: M, 1000 bp ladder Marker; Lane 1: Positive control (A. hydrophila MTCC 1739), Lane 2: Negative control (*Vibrio parahaemolyticus* ATCC AQ4037 isolates) Lane 3-5: Samples.

3.2. Scanning Electron Microscopy (SEM)

The scanning electron microscopy showed straight rods with rounded ends having single polar flagellum (Fig. 3A-B)

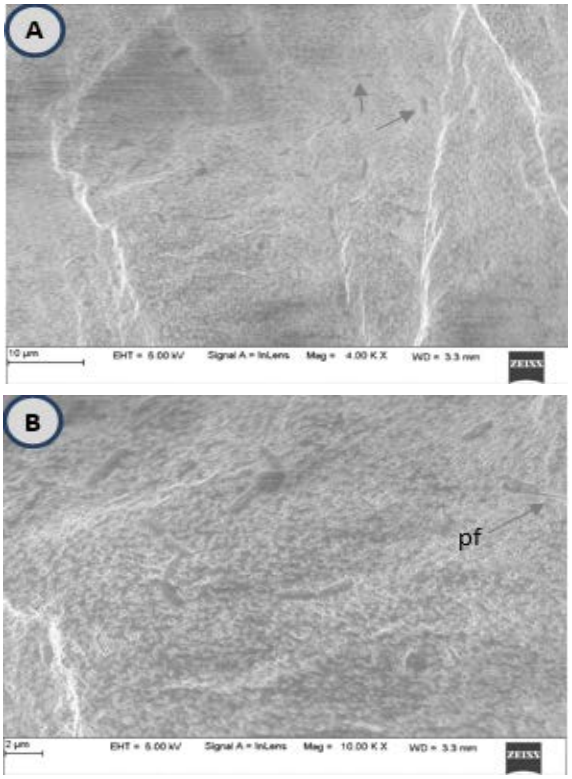

Figure 3. Feild emission scanning electron microscopy revealed **(A:4000X)** rod shapped *A. hydrophila* (arrows) **(B: 10000X)** with polar flagellum (pf)

3.3. Histopathological studies of infected organs

Several histopathological abnormalities in gill tissues, such as loss of secondary gill lamellae, lamellar clubbing, cellular necrosis and hypertrophy were observed (Fig. 4A-B). The liver histopathology displayed hepatocyte necrosis, pyknotic nuclei and formation of Kupffer cells (Fig. 5A-B). Loss of hematopoietic tissues, destruction of Bowman's space, tubule degeneration, glomerular necrosis, tubular necrosis, infiltration of leukocyte cells was detected in kidney section (Fig. 6A-B). Histopathology of spleen showed macrophages and lymphocytes aggregated around ellipsoids accompanied with hemosiderosis (Fig. 7A-B). Though intestinal architecture was not altered in infected pangasius, sloughed necrotic debris in lumen increased goblet cells was noted (Fig. 8A-B).

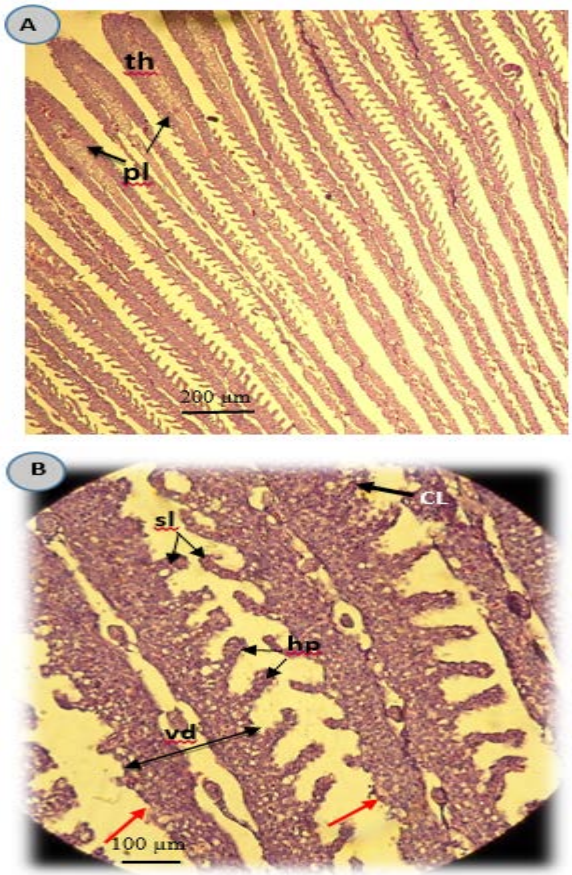

Figure 4. Photomicrographs of the gill of infected *P. hypophthalmus* (**A: 100x**), showing thickening (th) of primary gill lamellae (pl) **(B: 400x, H & E) C**lubbing, vasodialation (vd) and loss of secondary gill lamellae (sl, red arrows) accompanied with hypertrophy and hyperplasia

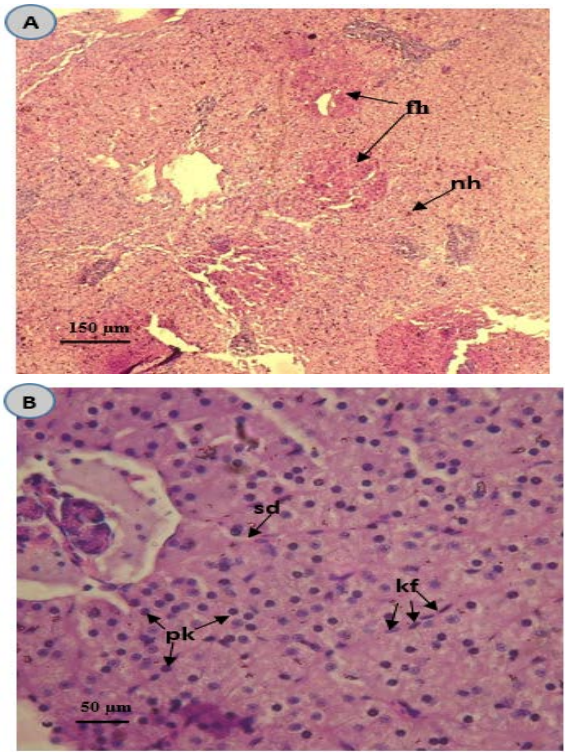

Figure 5. Liver histological section of naturally infected pangasius, display (**A: 100x**), multifocal haemorrhages (fh) with necrotized hepatocytes (nh) (**B: 400x)** Numerous pyknotic nuclei (pk), aggregated Kupffer cells (kf)

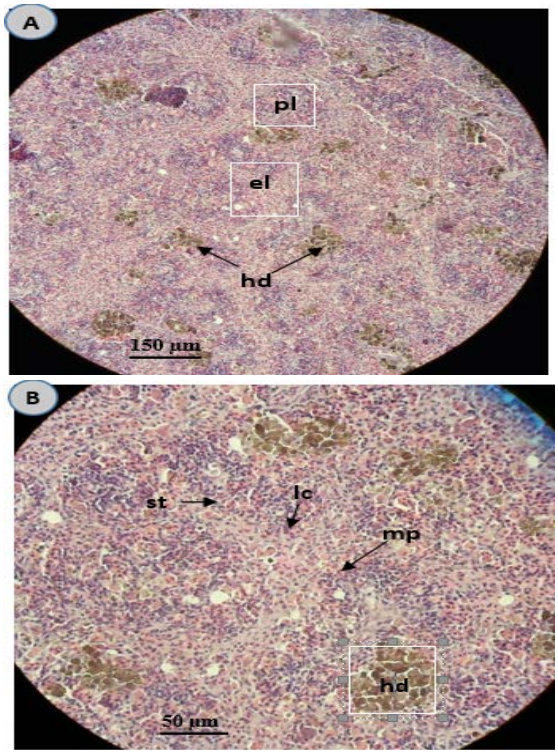

Figure 7. Photomicrographs of the infected spleen, **(A: 100x)** red and white pulp (pl) and ellipsoids (el) and numerous hemosiderosis (hd) **(B: 400x)** splenitis (st) with macrophages and lymphocytes aggregated around ellipsoids accompanied with hemosiderosis

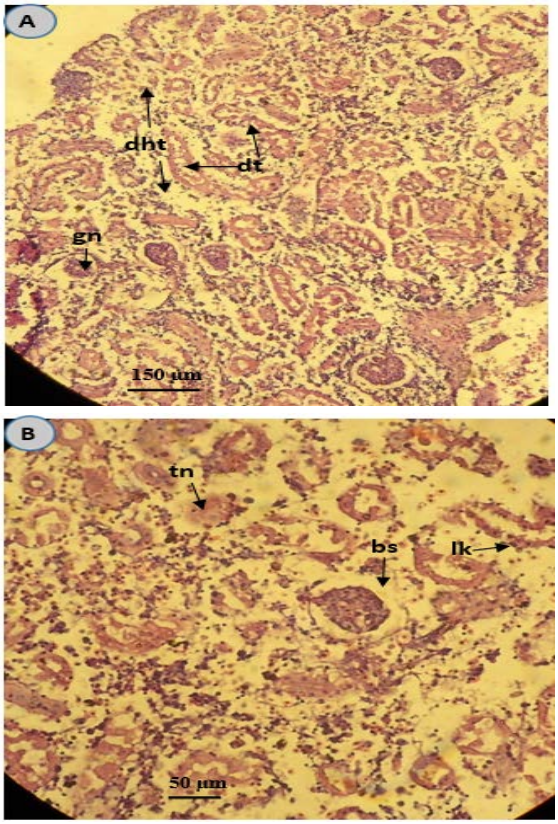

Figure 6. Kidney histopathological changes of infected P. hypophthalmus showing (A: 100x), acute degeneration of hematopoietic tissues (dht), degeneration of both distal and proximal tubules (dt) glomerular necrosis (gn) and (B: 400x) destruction of Bowman's space (bs), tubular necrosis (tn), necrotic tubules (lk).

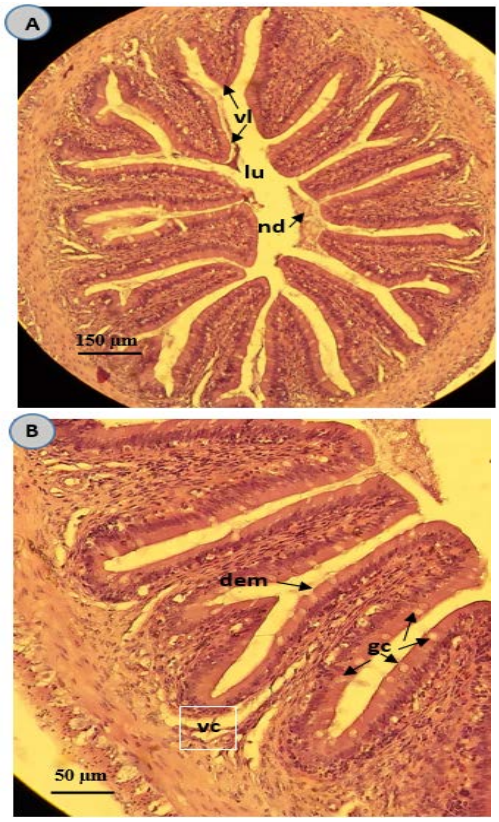

Figure 8. Intestinal histopathology of spontaneously infected pangasius, **(A:100x)** normal architecture of villi (vl) with sloughed necrotic debris (nd) in its lumen (lu) **(B: 400x)** vacuolation (vc), damage of enterocytes (dem) and increased aggregated goblet cells (gc).

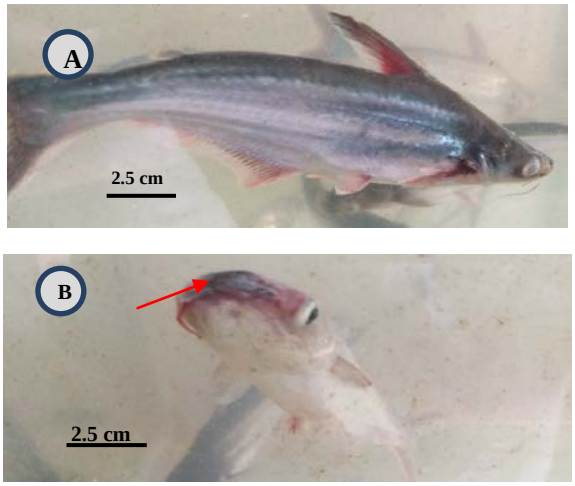

Figure 9. Juvenile pangasius were challenged with isolated *A. hydrophila* from naturally infected pangasius showing the **(A)** hemorrhagic fin bases **(B)** Reddish head with grayish (arrow) cotton wool patches on the tip of mouth and rectal protrusion.

3.4. Challenged studies

In challenged studies juvenile of pangasius showing the similar clinical signs (Fig. 9A-B) compared to the spontaneously infected adult *P. hypophthalmus* collected from earthen pond.

4. Discussion

Bacterial diseases are often regarded as the vicious circle in fish health management, in which administration of drug leads to many negative effects on living biota and may be exacerbated with the change of climate (Houston *et al.,* 2020). Among the infectious diseases, bacterial diseases accounted for more than half (54.59%) routine outbreaks of diseases in finfish aquaculture (Dhar *et al.,* 2014). Gram negative *A. hydrophila* infection known as 'motile aeromonas septicemia' is an opportunistic invader associated with epizootic ulcerative syndrome (EUS) and/or in stressful water environment (Roberts, 1993; Pathiratne *et al.,* 1994; Lio-Po *et al.,* 1998). Nowadays, traditional farmers are usually culturing pangasius catfish in an intensive system with frequent malicious aeromonads infection. This study was aimed to detect the causative agent and histopathological studies involved in the mass mortality of the pangasius in earthen pond of Karnataka, India.

There has been an increasing trend to use Rimler-Shotts (R-S) selective medium for the isolation pathogenic *A. hydrophila*. In the present study, yellow colonies were grown in overnight incubation of inoculated agar media. Rimlar-Shotts agar media supplemented with an antibiotic, novobiocin showed growth of yellow colonies on agar plates for the isolation of *A. hydrophila* from *Oreochromis niloticus* (Aboyadak *et al.,* 2017; AlYahya *et al.,* 2018), rohu (Siriyappagouder *et al.,* 2014), gold fish (Mamun *et al.,* 2019a), water samples (Davis and Sizemore 1981; Arcos *et al.,* 1988), frozen fish (Yogananth *et al.,* 2009) and from human (Khalaf *et al.,* 2005). Isolation of A. *hydrophila* through R-S medium was 94% valid for presumptive recognition in humans (Shotts and Rimler, 1973).

PCR based diagnosis are the confirmatory test for the identification of particular bacterial pathogens. In this study, we used specific primers for the haemolysin genes to confirm *A. hydrophila*. Haemolysin is the virulent factor of *A. hydrophila* (Gonzalez-Serrano *et al.,* 2002; Castro-Escarpulli *et al.,* 2003). Also, an amplified DNA product of the expected size of 130bp was obtained by PCR. Identifying *A. hydrophila* isolates through molecular technique (PCR) which targets the haemolysin gene is more efficient, reliable, and faster compared to biochemical test (Wang *et al.,* 2003) and has also been found extremely effective (Stratev *et al.,* 2016). Several studies were conducted on the identification of pathogenic *A. hydrophila* targeting virulent gene such as haemolysin, lip and aerolysin (Xia *et al.,* 2004; Kingombe *et al.,* 1999; Yogananth *et al.,* 2009). The scanning electron microscopy revealed straight rods with rounded ends having single polar flagellum. Our results are corroborated with Roberts et al (2012) who reported similar morphological features of *A. hydrophila*.

Harikrishnan and Balasundaram (2005) described many clinical symptoms such as reddish spot, abdominal dropsy, exophthalmia caused by aeromoniasis. In other studies, moribund fish samples exhibited rail and fin rots (Austin and Austin 1993), dermal ulceration and muscle necrosis (Bullock *et al.,* 1971) and hemorrhagic bacteremia (Leung *et al.,* 1995), is also evident in the present study. Motile aeromonas septicemia also known as hemorrhagic septicemia in aeromonads infection and diagnosed by several external symptoms such as red spot on the whole body, hemorrhagic fin bases, deep ulceration, rectal protrusion, peritoneal dropsy, sloughed off epidermis, pale gills, abrasive muscle and often bled in bottom of the dorsal fin (Harikrishnan and Balasundaram, 2005). The most prominent internal signs are swollen abdomen, anemic body and excess formation of body fluid which cause anal bulge resulting dysfunction to the inner organs (Jhingran and Das 1990). *A. hydrophila* were isolated and identified from diseased pangas (Parven *et al.,* 2020; Nahar *et al.,* 2016), stinging catfish (Goni *et al.,* 2020, Rashid *et al.,* 2008), Nile tilapia (Hamom *et al.,* 2020), Indian major carp (Lakshmanan *et al.,* 1986)

Histopathological section of gill tissue in the present study displayed several pathologies such as clubbing, disappearance of secondary gill lamellae, substantial hypertrophy, and hyperplasia at the nib of primary gill lamellae. Thickening of primary lamellae and vasodialation in approximately 90% of the area with congestion and lamellar joining in secondary gill lamellae was observed. Cellular necrosis, blood cells, congestion, hypertrophied and hyperplastic secondary gill lamellae were also seen from diseased pangasius. Gills are the first line of defense in fish, therefore recurrent bacterial infections resulted in significant changes in gill architecture and physiology. Channel catfish gills showed similar histopathological alteration such as fusion and thickening of gill lamellae after experimentally infected with virulent *A. hydrophila* (Abdelhamed *et al.,* 2017). Similarly, authors of a recent study revealed secondary gill lamellar loss in addition to clubbing and hyperplasia from farmed pangasius in Maharashtra, India (Kumar *et al.,* 2015). Histological changes offer a quick method to determine the effects of irritants, in various tissues and organs (Shraideh and Najjar, 2011).

The liver tissue sections of diseased pangasius revealed multiple hemorrhages on the hepatic tissues along with

numerous pyknotic nuclei, dilated sinusoids, vacuolization of cytoplasm, cellular necrosis of hepatocytes and aggregated Kupffer cells. Supplementation of lead acetate caused hepatic necrosis and degenerative liver cell as well as upregulated the Kupffer cells numbers in other vertebrates (Albishtue *et al*., 2020). Mamun *et al.,* (2019b) reported histopathological abnormalities in gill, liver, and kidney in pangasius fed *A. hydrophila* as oral vaccine. AlYahya *et al.* (2000) reported severe aggregation blood cells and accruement of pyknotic nuclei in the hepatopancreas of Blue nile tilapia artificially infected with *A. hydr*ophila. Focal necrosis with lymphocyte infiltration, presence of melanomacrophage centres accompanied with necrotic hepatocyte (Faruk *et al.,* 2012) indicated the cytotoxicity by *A. hydrophila* toxins (Donta and Haddow, 1978) could lead to the primary organ failure (kidney and liver). Histopathology of liver, kidney and spleen of natural infected barramundi was validated with severe pathologies such as macrophage aggregation with hepatitis, degeneration of hematopoietic tissues and granulomatous splenitis (Loach *et al.,* 2017).

Renal histopathology revealed acute degeneration of hematopoietic tissues, and destruction of Bowman's space, glomerular necrosis, tubular necrosis, infiltration of leukocytes was detected. Our findings were corroborated with the symptoms reported in other teleosts (Islam *et al.,* 2008; Kumar *et al.,* 2016; AlYahya *et al.,* 2000; Fergason *et al.,* 2001; Abdelhamed *et al.,* 2017). In the present study, among all organs the kidney become the most affected organ, and virtually lost its original anatomy. Fish kidney is a commonly targeted organ during bacterial sepsis due to the toxins released by aeromonads (Kumar *et al.,* 2015). Severe histopathological changes were noticed in kidney such as degeneration of renal tubule with alteration of renal corpuscle, necrotic capillaries and loss of Bowman's capsule when fish were exposed to polluted water (Takashima and Hibiya 1995). Invasion and multiplication of pathogenic bacteria can damage the kidney and lead to the glomerulonephritis (Prasad *et al.,* 2018).

Spleen is regarded as one of the vital organs that respond to fight microbial infection through innate and adaptive immune system. Extended ellipsoid with white and red pulp were observed in the present study. However, spleen section showed splenitis with aggregated macrophage cells and numerous hemosiderosis. During bacterial infection, macrophages congregate, resulting in erythrocyte phagocytosis in the spleen and iron deposition (hemosiderin), which leads to splenic hemosiderosis (Wang *et al.,* 2010). Spleen histopathology of experimentally infected channel catfish revealed degeneration of endothelial and reticular cells; in addition to pyknosis, cytolysis and accumulation of hemosiderosis (Abdelhamed *et al.,* 2017) is also evident in our study.

The gut morphology of the intestinal section did not alter in this study. However, several histological changes such as damage of enterocytes, vaculoation, necrotic enterocytes and infiltration of leukocyte cells were observed. Abundant goblet cells in the present study indicated excess production of mucus. The goblet cells are also known as mucous cells playing an important role in defense mechanism of host (Rathore *et al.,* 2019). It secrets the mucus to keep the gut environment healthy and protective (Cerezuela *et al.,* 2012). The goblet cells along with enterocytes can evoke the nonspecific immune system to enhance the phagocytosis process for the elimination of pathogenic bacteria from intestinal surfaces. Compared to the present findings, similar pathology such as vacuolation, necrosis, loss of villi and hemorrhages in mucosa and sub mucosal layers of experimentally infected *Heteropneustes fossilis* (Islam *et al.,* 2008) were also delineated. Similarly, histomorphology of gut of channel catfish revealed necrotized mucosal and enterocytes surface accompanied with bacterial accretion and enteritis (Abdelhamed *et al.,* 2017).

5. Conclusion

Aeromoniasis, known as motile aeromonas septicemia or red spot diseases, is one of the notorious problems in modern and traditional aquaculture farms in India. Acute *A. hydrophila* infection can cause a farmer to lose their entire crop. Early detection could provide the necessary clue for the prevention and disease management. The ubiquitous opportunistic pathogen, *A. hydrophila* has been potential threat to the pangasius farming, and care must be taken in order to control this disease with proper medications and prophylactic measures. Good aquaculture practices including effective water management in dry season can reduce the bacterial infection in traditional (earthen) pond aquaculture. The best weapon a farmer has to be follow is the basic biosecurity principles and adopting sustainable management practices towards maximum yield.

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Conflict of Interests

Authors have no conflict of interests to declare.

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Physiological Response and Detection of *Inh2* Gene in Dieng Red Potato (*Solanum tuberosum* L.) Affected by Frost

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Abstract

In this study, the response of Dieng red potato plants to frost exposure was evaluated physiologically by testing electrolyte leakage and chlorophyll content, and molecularly by detection of *Inh2* gene. The physiological response shows an increased electrolyte leakage rate and a significant decrease in chlorophyll content in plants exposed to frost compared to those grown in normal temperatures. Sequencing of *Inh2* gene identifies four homologous *Solanum tuberosum* alleles. The difference in the order of *Inh2* lies in the gap of 12 bp. Phylogenetic analysis shows that *inh2* gene is in a cluster with INH2α*C allele and has evolved slowly from a common ancestor with *S. lycopersicum*. This is the first study on the physiological mechanisms of frost tolerance and the sequencing of *Inh2* gene in Dieng red potatoes. Hopefully, this study will provide useful information for the breeding of low-sugar Dieng red potatoes.

Keywords: Dieng, electrolyte leakage, chlorophyll, *Inh2*, frost

1. Introduction

Potato (*Solanum tuberosum* L.) includes thousands of varieties that vary in size, shape, pigment, and other characteristics. One of the pigmented potatoes that has been widely cultivated is red potato plant. The glycemic index (GI) value of potato tubers varies widely and depends on the type, storage and serving. Red potato tubers have a lower GI value than other types of potato tubers. The low GI value in red potato tubers reaches 56 when served by boiling and consumed when cold (Eleazu, 2016). Red potato tubers also contain potential nutritional sources in the form of carbohydrates, minerals, vitamins C and B_1 (Beals, 2019). Therefore, choosing red potato tubers to serve as diet food is the best choice. Furthermore, there are pre-harvest factors that affect red potato tubers, namely the temperature of their growing period. Potato plants are very sensitive to frost, so temperature is one of the factors that affect the productivity of red potatoes.

Potato plants cannot tolerate low temperatures and will immediately show symptoms of freezing damage (Che *et al.*, 2020). Potato plants are not able to tolerate the formation of ice in its tissues, either extracellular or intracellular. Leakage of electrolytes from cells often occurs after frost injury (Arvin and Donnelly, 2007; Rooy *et al.,* 2017). Another damage that occurs after an electrolyte leak is a reduction in photosynthesis. Chlorophyll content of plants has a good correlation with the ability of photosynthesis. The reduction in chlorophyll contents in potato plants has been shown to slow down the rate of photosynthesis (Harb and Lahham, 2013; Li *et al.,* 2021).

Potato plants exposed to low temperatures will also cause the accumulation of reducing sugars, namely glucose and fructose or cold-induced sweetening, CIS (Datir *et al.,* 2019). Acid invertase activity was the most significant factor in determining the accumulation of fructose and glucose (Stein and Granot, 2019). Acid invertase activity can be controlled post translation by invertase inhibitors. Invertase inhibitor is of two types, the apoplastic invertase inhibitor encoded by *Inh1* gene and the vacuolar invertase inhibitor encoded by *Inh2* gene (Datir, 2020). According to Liu *et al.* (2010), the interaction between acid invertase and vacuolar invertase inhibitor may play an important role in controlling reducing sugars.

Understanding the response of potato plants to frost is essential for the development of cold-resistant crops. Red potatoes used in this research come from the Dieng. This is because air temperature in Dieng during the dry season (June-August) is very low and can reach freezing point in the morning, thus causing frost. The objectives of this study was to determine the physiological response of red potato plants to the amount of electrolyte leakage and chlorophyll content based on its resistance after exposure to frost and to analyze the sequence and compile the phylogenetic tree of *Inh2* gene of Dieng red potato plants. The results of this study are expected to be used as supporting data and a first step to breed cold-tolerant red potato plants so that they can be used as diet food by producing low-sugar potatoes through the mechanism of potato plants in inhibiting the formation of reducing sugar.

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2. Materials and Methods

The materials used in this study were a tuber and leaves of Dieng red potato obtained with permission from the Kejajar District Agricultural Extension Center. For determination of electrolyte leakage and chlorophyll content, plants were divided into three groups. Group one, a tuber was planted in a pot and grown at normal temperature 24/31° C (day/ night temperature) in conditions of long days (16 hours of light) and a relative humidity of 65- 95%. In the other two groups, plant leaves were taken directly in the field, namely in the Dieng area, and had been exposed to natural frost in the early hours. The two groups exposed to frost were taken at different altitudes, namely 2.110 m above sea level on the hill and 2.064 m above sea level in the valley. Leaf samples taken were immediately put in liquid nitrogen, stored in a styrofoam box filled with ice and testing was carried out immediately. For *Inh2* gene detection, healthy red potato leaves were selected, one month old, and very young leaves were taken from the shoot tips. The samples were put in a perforated ziploc bag and put into a styrofoam box filled with ice.

2.1. Determination of Electrolyte Leakage

The method for determining electrolyte leakage follows the method of Campos *et al.* (2003). Potato leaves were cut into discs with an area of 0.5 cm^2 in each group. The first group came from plants grown at normal temperatures, and the other two groups from plants that grew in nature and were exposed to frost. The leaf discs were then placed in 25 mL of demineralized water, incubated with a room temperature shaking incubator at 200 rpm for two hours. The electrolyte conductivity value C1 is measured with a conductivity meter (ExStih II, EC500, Extech, US). The sample was then heated in an oven for 30 minutes and cooled. The electrolyte conductivity value C2 was measured. Conductivity measurements are carried out 3-5 repetitions. The results of electrolyte leakage are expressed in relative conductivity (C%).

2.2. Determination of Chlorophyll Content

The method of determining chlorophyll content follows the method Liang *et al.* (2017). Potato leaves were prepared 100 mg from each group. The first group came from plants grown at normal temperatures and the other two groups from plants that grew in nature and exposed to frost. The leaves were crushed and homogenized at 10 mL of 80% acetone. Samples were centrifuged for 15 minutes at 10,000 rpm. The supernatant formed was then used to measure the absorbance at 633 nm and 645 nm against the solvent blank (acetone) using a spectrophotometer (UH5300 Spectrophotometer, Hitachi, Japan) with three repetitions. The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation from Arnon (1949).

2.3. Genomic DNA Isolation

Genomic DNA isolation from 0.5 g of red potato leaves. Genomic DNA was isolated using the modified Doyle and Doyle (1987) CTAB method. The quantity of DNA was determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific).

2.4. DNA Amplification

PCR Mix compositions were made in 50 µL. DNA was amplified using *Inh2* forward primer (5′-CCTTCATCAACTTCTCATTTCTTC-3'), *Inh2* reverse primer (5′-GTGCATTGAACGGCAAATTA-3′) (Datir *et al.,* 2019). PCR mix is made with Mytaq HS Red Mix as much 50 µL starting with mixing 25 µL Mytaq HS Red Mix; the forward and reverse primers each are 3 µL; DNA template, 2 µL; and ddH_2O, 15µL. The PCR conditions used were 30 seconds of pre-denaturation at 98° C, 15 seconds of denaturation at 98° C, 30 seconds of annealing at 55° C, 30 seconds of elongation at 72° C, and 10 minutes of post-elongation at 72° C carried out in 35 cycles. The PCR product was then subjected to electrophoresis 1% agarose (1x TAE buffer), stained with florosafe and visualized with Gel documentation XR+ system (Bio-Rad).

2.5. DNA sequencing

Analysis of potato DNA sequences using the company 1st BASE and Genetics Science services. The results of the sequences were then analyzed to compare the similarities of sequences available to GenBank through the Basic Alignment Search Tool (BLAST) on NCBI. The putative mRNA and protein sequence using MEGA X and Geneious Prime software. Phylogenetic analysis uses the molecular application ClustalX to align the sample sequences with other organism sequences available on the NCBI website and the MEGA X application to construct phylogenetic trees.

3. Result

3.1. Electrolyte Leakage

In this study, potato plants exposed to frost were taken in two different places, namely at an altitude of 2,100 m above sea level i.e. on a hill and 2,064 m above sea level, i.e. in a valley. Potato sampling in two different places was based on a research done by Chung *et al.* (2006) that cold air from the slopes will descend the slopes and settle in the valley. As a result, frost zones will tend to accumulate at the bottom of the valley. So, it is expected that potato plants that grow in valleys will show higher electrolyte leakage due to the amount of frost compared to potato plants that grow on hills with little frost.

The conductivity test showed that the leaf electrolyte leakage increased due to exposure to frost as shown in Figure 1. There is a significant difference at normal temperature, and the average electrolyte leakage value is around 39,4%. Potato plants exposed to frost in the valleys showed an increase in electrolyte leakage of 96,4%, while potato plants in the hills only reached 85,8%.

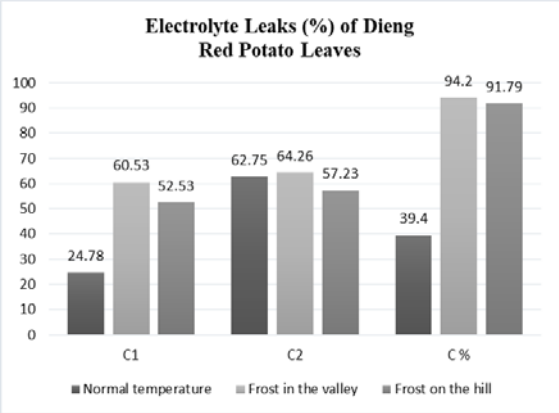

Figure 1. Results of electrolyte leakage (%) leaves of Dieng red potato plant leaves due to frost exposure, and plants not exposed to frost. Information: C1= initial conductivity value, C2 = conductivity value after heating and C% = relative conductivity value

3.2. Chlorophyll content

Plants optimize light absorption and photosynthesis through adequate chlorophyll content. Stress at low temperatures is known to affect photosynthesis and reduce the ability of plants to absorb light (Liu *et al.,* 2013). The results of determining the chlorophyll content of potato plants in Figure 2 exposed to frost both in the valley and the hill showed low chlorophyll contents compared to those of potato plants that were not exposed to frost; namely chlorophyll contents reached 7,21 mg.g^{-1}FW. Chlorophyll contents were seen to decrease in potato plants in the valley, namely 1,01 mg.g^{-1}FW compared to those in the hill, namely 1,79 mg.g^{-1}FW.

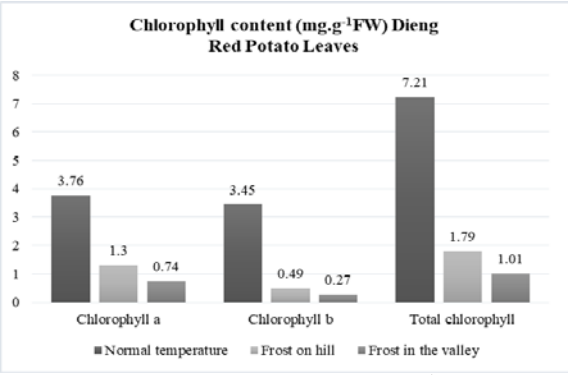

Figure 2. Results of chlorophyll content (mg.g^{-1}FW) leaves of Dieng red potato plant leaves due to exposure to frost, and plants not exposed to frost

3.3. The Inh2 Gene

The amplification results show good DNA quality with clear bands. The agarose gel showed an invertase inhibitor PCR amplicon of about ± 600 bp (Figure 3). The size of the potato *S. tuberosum inh2* gene varies widely. The result of DNA amplification in this study is by the report of Datir *et al.* (2019) and Liu *et al.* (2010). Therefore, the actual size of the *Inh2* gene in this study needs to be proven by means of amplified *Inh2* sequencing.

The purpose of *Inh2* gene sequencing is to determine the sequence of nucleotide bases and the size of the gene. The sequencing results are then combined, namely the unification of the primary forward *Inh2* and reverse *Inh2* primary sequences to obtain a complete sequence. The results of the second contig of *Inh2* primers showed that the length of the nucleotide base of the *Inh2* gene was 630 bp (Figure 4). The sequencing results were then aligned using the NCBI Blast Alignment Search Tool (BLAST) to determine the percentage of homology of the red potato *Inh2* gene base sequence obtained with the *Inh2* gene sequence database contained in the GenBank (Table 1). The BLAST results show that there are 11 *Solanum* species that have a maximum of 613 nucleotides out of 630 nucleotides.

Probability of mRNA and protein prediction were analyzed using MEGAX and Geneious Prime. The INH2 sequences of Dieng red potatoes were aligned and compared with our identified *Inh2* homology (Table 1). The next analysis is to compile a phylogenetic tree which aims to determine the kinship of a species, the amino acid sequences of the *Inh2* homologs identified by us (Table 2). The ClustalX software is used for aligning sequences and the software for reconstructing phylogenetic trees. The phylogenetic tree formed in Figure 5 consists of four species (Table 2), namely Dieng red potato, *S. tuberosum*, *S. lycopersicum* and *Manihot esculenta* as an outgroup. Dieng red potatoes are in the same clad with *S. tuberosum* for INH2α* C. Dieng red potato along with *S. tuberosum* and other wild potatoes appear to have ancestry from *S. lycopersicum* (Slugina *et al.,* 2020). Genetic distance shows the kinship relationship between each sequence. The smaller the number of genetic distances, the more linked the sequence is. The genetic distance results of *Inh2* gene showed that Dieng red potatoes were closely related to the *S. tuberosum* INH2alpha* C and INH2alpha* D alleles with a genetic distance of 0.054 and 0.060. This was further followed by S. *tuberosum* INH2alpha* A, INH2alpha* B.

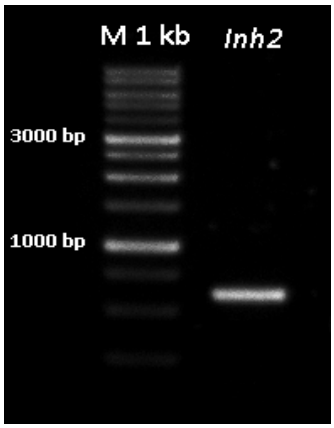

Figure 3. Visualization of PCR products from the Dieng red potato plant of *Inh2* geneDescription: Line 1; Marker 1 kb; Line 2; Dieng red potato *Inh2* gene

```
  1 ccttcatcaa tttctcattt tttcaatttt caaaaaaaaa aagttaaaaa aatggagaaa
 61 tttattcccc atattgatgc taatcaccaa tttggcactc aacaacgata acaacaacaa
121 caacaataat tataatctca tacactcaac gtgtagggag accccatatt actccctatg
181 tctcaccacc ctacaatccg atccacgtag taacgaggtt gagggtgatg atgccatcac
241 caccctaggc ctcatcctgg tggacgcggt gaaatcaaag tccatagaaa taatggaaaa
301 aataaaagag ctagagaaat cgaaccctga gtggcgggcc ccacttagcc agtgttacgt
361 ggcgtataac gccgtcctac gagctgatgt aacggtagcc gttgaagcct taaagaaggg
421 tgtccctaaa tttgctgaag atggcatgga tgatgttgtt gttgaagcac aaacttgtga
481 gtatagtttt aattattata ataaatttgga ttttccaatt tctaatttga gtagggaaat
541 aattgaacta tcaaaagttg ctaaatccat aattagaatg ttttttagaa gggggaaaaa
601 aaagtttggg gtttaatttg ccgttcaatg
```

Figure 4. The sequence of *Inh2* gene in Dieng red potato plant

Table 2. BLAST results of Dieng red potato plant *Inh2* gene on NCBI

Organism	Description	Query coverage	Percent identity	Accession
S. tuberosum	*Inh2alpha*C*	100%	97,30%	FJ810208
S. tuberosum	*Inh2alpha*D*	99%	97,29%	FJ810209
S. tuberosum	*Inh2alpha*B*	99%	95,44%	FJ810207
S. tuberosum	*Inh2alpha*A*	99%	95,30%	FJ810206
S. lycopersicum	*Inh2*	92%	88,85%	NM_001329220

Table 3. Predictions of amino acid composition, molecular weight and isoelectric point of Vacuolar Invertase Inhibitor

Organism	Description	Amino acid	Molecular weight	Isoelectric point	Accession
Dieng Red Potato	*Inh2*	179	20.28	5.3	
S. tuberosum	*Inh2α*A*	181	20.51	4.57	ACO35697
S. tuberosum	*Inh2α*B*	180	20.37	4.69	ACO35697
S. tuberosum	*Inh2α*C*	178	20.14	4.57	ACO35697
S. tuberosum	*Inh2α*D*	178	20.13	4.47	ACO35697
S. lycopersicum	*Inh2*	175	19.90	5.00	ACO35697

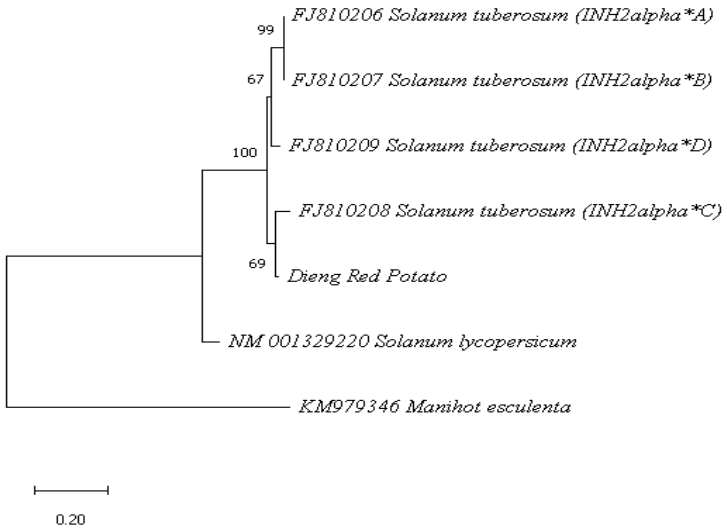

Figure 5. Phylogenetic tree of Dieng red potato *Inh2* gene

Table 4. Genetic range of Dieng red potato *Inh2* gene with several *Solanum* comparators from NCBI.

No	Species	1	2	3	4	5	6	7
1	*S. tuberosum Inh2alpha*A* (FJ810205.1)							
2	*S. tuberosum Inh2alpha*B* (FJ810207.1)	0,000						
3	*S. tuberosum Inh2alpha*C* (FJ810208.1)	0,123	0,123					
4	*S. tuberosum Inh2alpha*D* (FJ810209.1)	0,060	0,060	0,118				
5	*M. esculenta* (KM979346.1)	1,543	1,543	1,530	1,507			
6	*S.lycopersicum* (NM001329220.1)	0,270	0,270	0,280	0,298	1,373		
7	Dieng Red Potato	0,073	0,073	0,054	0,060	1,596	0,254	

4. Discussion

In this study, Dieng red potatoes affected by frost were analyzed physiologically and molecularly. Physiologically, electrolyte leakage and chlorophyll content were estimated. When plants experience frost stress, the cell membrane structure will be damaged. The level of damage to the cell membrane can be observed from the electrolyte leakage value using a conductivity meter. Relative conductivity is an effective indicator to evaluate the responsiveness of plants to low temperature stress (Liu *et al.,* 2013). Potatoes exposed to frost cause the cell membrane to change from a relatively liquid state to a more rigid state, resulting in reduced membrane permeability. For such experiments, the plants are subjected to frost compared to normal temperatures. The relative electrolyte leakage of leaves increases rapidly in the presence of frost as shown in Figure 1. Research on electrolyte leakage in potato plants due to low

temperatures was started by Lindstrom and Carter (1985). Temperatures -4 ° C indicates a large number of electrolyte leakage (80-90%) for each potato crop compared to controls stored at 0° C. In 2007, Arvin and Donnelly reported that the average value of potato electrolyte leakage due to cold stress was 61,9% for several cultivars of S. *tuberosum* and reached 47,9% for wild potato species. Plasma membrane is considered to be the site of the main attack when cold injury occurs (Huang *et al.,* 2014). As a result, lipids in the plasma membrane will undergo a transition phase. Liquid crystals or the formation of liquid into a gel or solid will affect the membrane permeability when the temperature drops (Huang *et al.,* 2014; Al-Shuneigat *et al.,* 2015).

Injury due to low temperatures also causes a decrease in chlorophyll content thereby affecting plant photosynthesis (Liu *et al.,* 2013). In plants that are cold tolerant, a decrease in photosynthesis will affect cold acclimation and prevent the attainment of maximum freezing tolerance. This is because the cold acclimation process requires a lot of energy due to changes in metabolism resulting from exposure to low temperatures. Potato plants are very sensitive to frost and cannot tolerate coldness (Chang *et al.,* 2014). As a result, there will be a decrease in chlorophyll content (Figure 2). The decrease in chlorophyll content of leaves exposed to frost in the valley was higher than that of the leaves on the hill. This is suitably described Chung *et al.* (2006) that the valley floor area accumulates more frost, so that the presence of frost causes the accumulation of chlorophyl to be low. Results of determination of potato chlorophyll content in this study are in line with research published previously by Li *et al.* (2021) which showed a similar reduction in chlorophyll content after exposure to low temperatures. The results of research by Li *et al.* (2021) showed that the total chlorophyll content decreased with increasing treatment duration, indicating that chlorophyll synthesis was inhibited during low temperatures.

Inh2 gene encodes the vacuolar invertase inhibitor protein that plays a role in inhibiting the activity of invertase acid. The BLAST results show the maximum percent identity is 97,30%, and the query coverage is 100%. Dieng red potato *inh2* gene along with the alleles INH2alpha* A, INH2alpha* B, INH2alpha* C, and INH2alpha* D of the species *S. tuberosum* shows similarities to the sequence starting at base 42. The difference in the next *Inh2* sequence lies in the gap of 12 bp. Analysis of the predicted protein sequences in Dieng red potatoes showed 178 amino acid residues with a calculated molecular mass of 20.28 kDa and an isoelectric point of 8,866. The INH2 protein prediction of *S. tuberosum* did not show different variations; INH2alpha*A (181aa, 20,51 kDa), INH2alpha*B (180aa, 20,37 kDa), INH2alpha*C (178aa, 20,14 kDa), INH2alpha*D (178aa, 4,47 kDa) with isoelectric point values between 4,47 to 4,69. The high isoelectric point value in Dieng red potatoes is due to the presence of amino acid groups, namely lysine, arginine and histidine which are incompatible with *S. tuberosum*. The isoelectric point value is strongly influenced by the ionized amino acid groups, namely arginine, aspartate, cysteine, histidine, glutamate, lysine, and glutamate (Mohanta *et al.,* 2019). The point of electricity of a protein is very important in understanding the biochemical function of proteins. Phylogenetic tree shows groupings with a bootstrap value of 10000 (Figure 5). The *Inh2* gene was first sequenced in the tobacco plant (Greiner *et al.,* 1998). Currently, the *Inh2* gene has been identified in many plants, such as *Arabidopsis* (Link *et al.,* 2004), corn (Bate *et al.,* 2004), soybeans, sweet potatoes, rice, and tomatoes (Rausch and Greiner 2004; Tymowska-Lalanne and Kreis, 1998). The *Inh2* gene is found in all parts of potato plant. During cold storage on potatoes, vacuolar invertase inhibitors accumulate in CIS-resistant cultivars rather than susceptible cultivars. Increasing the amount of vacuolar invertase inhibitor can contribute to suppressing the activity of invertase acid and preventing the cleavage of sucrose (Datir *et al.,* 2019).

5. Conclusion

Dieng red potato is a crop that is sensitive to frost. Frost injury causes changes in membrane fluidity and decreased photosynthetic capacity. This study shows that the exposure to frost led to increase in electrolyte leakage and decreases leaf chlorophyll levels. Plants that have been exposed to frost in the valley and on the hill show no significant value for physiological changes. This study provides an understanding of the physiological responses of Dieng red potato plants after exposure to frost on a field scale. Further research should include other physiological parameters on a laboratory scale.

The *Inh2* gene can be found in the Dieng red potato plant and evolutionary analysis shows that *Inh2* Dieng red potato is in the same clad as *S. tuberosum*, so its role is very important in inhibiting invertase acid activity and regulating sucrose metabolism. Studying the diversity of *Inh2* gene sequences is very important, especially Dieng red potatoes. This is because the demand for Dieng potatoes will increase every year so that a good cultivar is needed, especially one that can cope with stress due to frost. It is hoped that our research results contain information that can be used further by plant breeders to develop good potato cultivars.

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Antibacterial Activity of The Fungal Metabolite *Trichoderma longibrachiatum* against Multidrug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Extracts from sponge-associated fungus *Trichoderma longibrachiatum* have been studied and contain antibacterial compounds which can inhibit several pathogenic multidrug-resistant organisms. This study aims to determine the active fraction of the extract which is antibacterial against the gram-negative Multi Drug-Resistant *Klebsiella. pneumoniae* pathogen and gram-positive Methicillin-Resistant *Staphylococcus aureus*. In this study, the fungus was cultivated using solid media of malt extract agar (MEA) for 6-9 days (24 hours dark, static, pH 5.6, 60 ‰ salinity, and 27 ^{o}C). The mycelia and media were macerated by methanol and then partitioned using ethyl acetate. Active fraction tracing was carried out using the bioautography method and then isolated by the open column chromatography method. Antibacterial activity testing was done using the Broth Dilution method to determine the Minimum Inhibitory Concentration (MIC). The results of the study showed that ethyl acetate extract contained one active fraction (R_f value = 0.14), which has reactive characteristics on vanillin reagent and absorbed ultraviolet light (λ 375.5 nm absorbance peak). The active fraction was able to inhibit the growth of MDR *K. pneumoniae* and MRSA bacteria at the same MIC value, i.e. 256 µg mL^{-1}. In conclusion, an active fraction of *T. longibrachiatum* can be developed as an antibacterial against MDR *K. pneumoniae* and MRSA.

Keywords: Sponge-Associated Fungus, Active Fraction, Antibacterial, Minimum Inhibitory Concentration

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is an opportunistic pathogen which can be categorized in gram-negative group, non-motile, facultatively anaerobic, and rod-shaped bacterium. These bacteria produce Extended Spectrum β-Lactamases (ESBL) which can degrade certain antibiotics (β-lactam group), such as penicillin and cephalosporin, so they become inactive (Farhat *et al*., 2009). Also, it is protected by a capsule (composed of polysaccharides), both of which will further increase its pathogenicity. The infectious diseases caused by them are such as liver abscess, bacteremia, lung infection, acute leukemia, meningitis, and the bacteria may even cause death (Turton *et al*., 2010; Adwan *et al*., 2020)). Hospitalized patients with weak immunity are the main target for this bacterial attack. In current conditions, there is a tendency to increase the prevalence of infection caused by *K. pneumonia* along with the decrease in sensitivity to antibiotics used to treat the infection (Li *et al.*, 2014; Santana *et al*., 2016). According to Adwan *et al*. (2020), the prevalence of capsular polysaccharide genes among *K. pneumoniae* and high level of drug resistance will make bacterial infections are increasingly widespread, both in the hospital environment and community which leads to failure of treatment.

Staphylococcus aureus (*S. aureus*) is another cause of some dangerous infection. *S. aureus* is gram-positive, coccus-shaped, non-spore-forming, non-motile, facultative anaerobes, and forms a biofilm. In particular, biofilm formation by Methicillin-Resistant *Staphylococcus aureus* (MRSA) infection makes difficult treatment and causes a hard prognosis (Sato *et al*., 2019). For a long time, the infection has been treated by such semi-synthetic penicillin antibiotics as methicillin, and it is considered the first representative of multidrug-resistant bacteria (MDR) since 1961s (Gajdács, 2019). MRSA incidence and prevalence are more likely to occur in health care/hospital settings area than in the community environment. These bacteria produce enterotoxins (exotoxins), ESBL enzymes, and immune modulators (Abbas *et al*., 2015).

According to Narendran and Kathiresan (2016) as well as Basiriya *et al.* (2017), some species of *Trichoderma* sp. have been screened and eventually have the ability to synthesis some antibacterial compounds. Moreover, some studies reported that ethyl acetate extract of *Trichoderma* sp. has antibacterial activity against pathogens (*Pseudomonas aeruginosa, Escherichia coli, Bacillus cereus,* and *S. aureus,*). These fungi isolated from the

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mangrove rhizosphere could be used as a producer of secondary metabolites to be developed into a new antibiotic against resistant bacteria. This was also stated by Synytsya *et al.* (2017) who investigated antibacterial compounds derived from ethanol and petroleum ether extracts. Some other researchers stated that the potential of antibacterial compounds produced by *Trichoderma* sp., such as *trichodin* A and B, pyridoxatin have antibacterial activity against *Staphylococcus epidermidis* and *S. aureus* (Wu *et al.*, 2014; Wang *et al*., 2020), and trichodermaquinone to be antibacterial compound against MRSA (Khamthong *et al*., 2012). There are many classes of secondary metabolite considered as antibacterial compounds from marine fungi, such as glycopeptides, peptides, proteins, lipopeptides, aminolipopeptides, polyketides, polybrominated biphenyl ether, cyclic depsipeptides, terpenes, pentaketides, alkaloids, diketopiperazins, anthraquinones, chromones, steroids, lactones, quinolone derivatives, trisindole derivatives, macrolactam, and phenol derivatives (Thomas *et al*., 2010; Nalini *et al*., 2018; Wang *et al*., 2020).

A review by Li *et al.* (2019) showed that *Trichoderma* spp. can produce many metabolites with different bioactivities. These fungi are commonly distributed in many ecosystems, including the sea. The investigation by Sedjati *et al.* (2020) proved that ethyl acetate extract of sponge-association fungus *T. longibrachiatum* contains compounds that have antibacterial activity against MRSA and *K. pneumoniae*. Based on these findings, this study aims to determine the active fraction in ethyl acetate extract of *T. longibrachiatum* using bioautographical methods by the guidelines of its bioactivity test results.

2. Materials and Methods

2.1. Fungus Isolate

The sample used in the study was from the sponge-associated fungus obtained from Falajava Beach, Ternate Island, North Maluku, Indonesia (00°47'09.12" N; 127°23'21.76" E coordinates) with TE-PF-03.1 code. The fungi have been identified molecularly using Internal Transcribed Spacer (ITS) rDNA sequence, and confirmed as *T. longibrachiatum* macro and microscopically (Sedjati *et al*., 2020).

2.2. Bacterial Pathogen

The test bacteria used in this experimental study were MDR *K. pneumoniae* obtained from Microbiology Laboratory, Diponegoro National Hospital, and MRSA from the University of Indonesia. Before being used for antibacterial tests, pathogenic bacteria were recultured first. The process was done by taking bacterial stock colonies and transferring them into Mueller-Hinton Broth (MHB; Oxoid) and further incubated at 37°C for 24 hours.

2.3. Fungus Cultivation

Fungus cultivation according to the method by Sedjati *et al*. (2020). *T. longibrachiatum* isolate coded TE-PF-03.1 was subcultured using Malt Extract Agar (MEA; Merck). Then, the mycelia were taken about 2 mm in diameter and cultivated on new MEA media. The treatment of cultivation periods was carried out at 6,7,8, and 9 days (static, 24 hours in dark, pH 5.6, salinity 60‰, temperature 27°C). MEA preparation was conducted by using sterile seawater (solid, 20 mL media/Petri dish).

2.4. Extraction and Determining Extract Weight

After the cultivation period finished, the media and the mycelia were cut into small pieces and then macerated with methanol (1:1v/v), filtered using Whatman paper no. 42 and the filtrate were evaporated at a rotary evaporator with 40°C and low pressure. Furthermore, partitioning of the fungal extract was done using methanol-distilled water (50%) and ethyl acetate (1:1v/v). Moreover, each fraction was evaporated using rota vapor to be methanol and ethyl acetate extracts, and then these were weighed.

2.5. Profiling of Secondary Metabolites

The Thin Layer Chromatography (TLC) method (Harborne, 1984) was used for profiling chemical extracts. There were in total 10 µl of extract solution in methanol (1 mg mL^{-1}) was spotted on the baseline of the TLC plate (Merck, silica gel 60 F254). The mobile phases used were sequential based on polarity levels, i.e. a mixture of n-hexane and ethyl acetate (4:1; 3:2; 2:3; 1:4, and 0:5). Spot identification using the value of R_f (Retention Factor) and spraying with staining reagents. After the elution process, TLC was visualized by UV light (365 nm), 2% vanillin-H_2SO_4, 0.25% ninhydrin in acetone, and 1% ferric (III) chloride in methanol (Harborne, 1984; Sen *et al*., 2012; Trianto *et al*., 2019). Furthermore, the TLC plate was heated at 110 °C for 2-3 minutes. The same method was used to detect the active antibacterial fraction after the isolation process, along with an additional absorption profile against UV light (λ200-400 nm) using a UV-Vis spectrophotometer.

2.6. Antibacterial Activity Test

The antibacterial activity test was conducted using a disc diffusion assay method to determine the inhibition zone against pathogen growth. Pathogenic bacteria were cultured on Mueller-Hinton Agar (MHA; Oxoid) with an initial density equivalent to 0.5 McFarland (1.5 $x10^8$ CFU mL^{-1}). Extract in the dimethylsulfoxide (DMSO) solvent was tested against pathogenic bacteria. 10 µL of extract solution was dropped onto the sterile disc paper (6 mm diameter; Oxoid) with 500 µg $disc^{-1}$ concentration. The negative control used was DMSO, while the positive control used was chloramphenicol (30µg $disc^{-1}$; Oxoid). The inhibition zone was measured after 24 hours of incubation at 37 °C (Trianto *et al*., 2017).

2.7. Bioautography Test

The bioassay was done using contact techniques (Contact Bioautography) as the method done by Sakunpak and Sueree (2018) with minor modifications. Pathogenic bacteria were cultured on MHA media with 1.5 $x10^8$ CFU mL^{-1} initial density. The extract in ethyl acetate solvent (10 µL, 10 mg mL^{-1} concentration) was spotted on the TLC surface baseline and eluted with a suitable mobile phase to produce perfectly separated spots. The TLC plate was applied with silica surface attached to the MHA media surface (facing downward) and left for 60 minutes. Furthermore, the TLC plate was removed from the test bacteria medium and the Petri dishes were closed. All processes were carried out in a laminar flow cabinet. The incubation was carried out for 24 hours at 37 °C and the formation of the inhibition zone around the TLC spot was

carefully observed. The spots around which the next inhibition zone appears were called an active fraction.

2.8. Active Fraction Isolation

The active fractions found were isolated using the open column chromatography method with the appropriate mobile phase (referring to the TLC profile). The column was filled with silica gel (60–120 mesh) mixed with n-hexane: ethyl acetate (2:3) solvent. The extract was dissolved in the solvent and was slowly being loaded on the top surface of the silica gel. The extract was then eluted using a solvent sequence based on the polarity increase. Furthermore, the eluate coming out was collected using a test tube (every 10 mL of eluate), checked again using the TLC method, and the same eluates were put together. The eluates containing active fraction were concentrated at a rotary evaporator for further testing.

2.9. Minimum Inhibitory Concentration Test

Minimum Inhibitory Concentration (MIC) test was conducted based on the Broth Dilution method. MIC determination refers to the method proposed by Sowjanya *et al.* (2015) and Fajarningsih *et al.* (2018) using 96-well microplates with resazurin (Sigma-Aldrich) as an indicator of the viability of the test bacteria (REMA assay). A total of 100 µL of the extract solution in the DMSO solvent with the highest concentration (2,048 µg mL^{-1}) was filled in the first well in certain rows. The next well was filled with 50 µL of sterile MHB nutrients. The 50 µL test material was transferred from the first well to the next well to reach serial dilution (at wells no. 1-10). Then, 30 µl of resazurin solution (0.02% in distilled water) was added to each well. At last, 10 µl of the bacterial suspension (1.5 $x10^8$ CFU mL^{-1}) was added to each well. Chloramphenicol was used as a positive control (the highest concentration was at 64 µg mL^{-1}) and DMSO as a negative control (at well no. 11). The well contained MHB without extract as growth media control (at well no. 12). The microplate was incubated at 37 ^oC for 24 hours. After the incubation period, the well functioning as growth control would appear pink. The MIC value was determined based on the lowest concentration which could inhibit the growth of the tested bacteria.

3. Results and Discussion

3.1. Assessment of Antibacterial Potential

T. longibrachiatum species is one of the fungi species which are easy to cultivate. It can grow well in MEA and modified media. The modified media is prepared by replacing malt extract with a cheaper material, namely fish and cassava extracts. All of them produce secondary metabolites that have antibacterial activities, but the best is obtained from ethyl acetate extract of fungus cultivated with MEA. The best antibacterial potential achieved is against pathogens *K. pneumoniae* and MRSA (Sedjati *et al.*, 2020). Data from the results of this study indicated that the secondary metabolite product of *T. longibrachiatum* cultivated in MEA was mostly in the form of methanol extract (polar compound) and only a small part of them was ethyl acetate extract (semi-polar to non-polar compound). The weight of fungal extract is based on the polarity of its secondary metabolites after 6-9 days cultivation periods described in (Figure 1a). Ethyl acetate extract was produced in only small amounts. On the other hand, the ethyl acetate extract has antibacterial activity against these two pathogens as shown in (Figure 1b). The greatest potential as an antibacterial was seen in the ethyl acetate extract from the fungus which had been cultivated for 9 days.

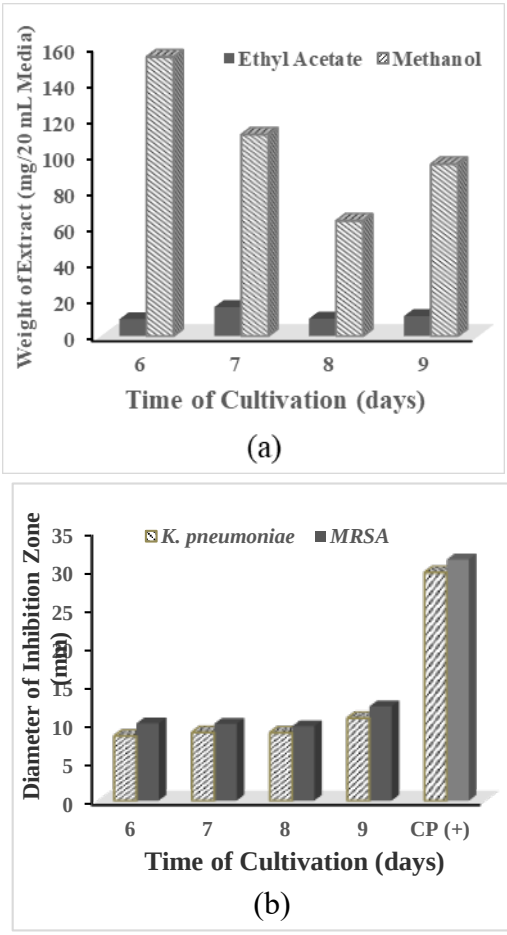

Figure 1. Characteristics of *T. longibrachiatum* extracts: (a) Extract weight on polarity basis; (b) Antibacterial potential of ethyl acetate extract against MDR pathogens *K. pneumoniae* and MRSA at a concentration of 500 µg $disc^{-1}$ (note: CP= Chloramphenicol, at a concentration of 30 µg $disc^{-1}$)

Some synthesized fungal secondary metabolites are only in small amounts because they are not for the main energy supply needed by the fungus and are only made at suboptimal conditions as a response to environmental pressure (Nielsen and Nielsen, 2017). The peak of secondary metabolite production in this study occurs when the fungal life cycle was in a stationary phase. This statement conforms to several research results stating that the fungus has entered a stationary period on day 6 to 9 after being cultivated (Gliseida *et al.*, 2013; Arumugam *et al.*, 2015). Methanol extract seemed to predominate over the extract of *T. longibraciatum.* However, when tested for antagonists against *K. pneumoniae* and MRSA at a concentration of 500 µg $disc^{-1}$, they did not show antibacterial activity. This fact is similar to the research result of Leylaie and Zafari (2018). In general, the ethyl acetate extract metabolite of the *T. longibrachiatum* is more likely to be antibacterial than its methanol extract. According to the statement of Chamekh *et al.* (2019), methanol extract is presumed to contain enzymes

synthesized by *T. longibrachiatum* for external digestion, along with several units of saccharides, amines/peptides, fatty acids/glycerol which are hydrolysis results of organic compounds in the media. Polar metabolites dissolved in methanol consist of enzymes (such as amylase, protease, and lipase) which are synthesized by fungi to degrade the nutrients in the media. Based on the research of Massadeh *et al*. (2010), the fungi can utilize a variety of carbon sources and produce various ligninolytic and cellulolytic enzymes. Added by Muthulakshmi *et al*. (2011), protease is produced by fungi from the first day of cultivation and reaches its peak on the 7th day (wheat bran as a media, pH 5.0, temperature 30^{0}C)

3.2. Secondary Metabolite of Fungal Extract

Chemical compounds contained in ethyl acetate extract of *T. longibraciatum* which was cultivated for 9 days can be traced based on its TLC profile as shown in Figure 2. Only ethyl acetate extract was used for the next stage of research since methanol extract is not potentially antibacterial. The best spot separation was seen in the results of TLC with a mobile phase of n-hexane and ethyl acetate (2:3) as seen in Figure 2. Based on the number of spots that appeared, at least 5 compounds were detected with R_f values: 0.14, 0.26, 0.57, 0.71, and 0.89. As congenial with the order of R_f values, the compound with the smallest R_f value is relatively the most polar, while the largest R_f is the most non-polar.

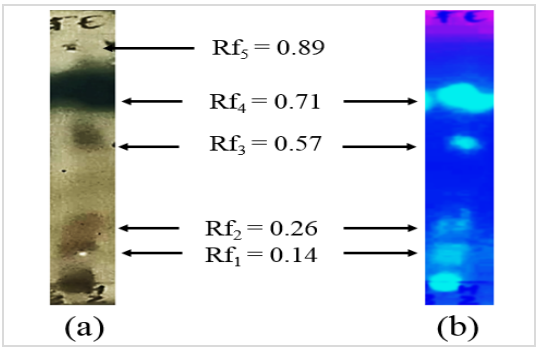

Figure 2. TLC Profile of ethyl acetate extract using mobile phase of n-hexane and ethyl acetate (2:3) : (a) Visualization results with 2% vanillin-H_2SO_4; (b) Visualization results with 365 nm UV light

Compound prediction in ethyl acetate extract of *T. longibrachiatum* was traced based on previous studies' references. The spot looks fluorescent blue when exposed to UV light indicating that the organic compound has a double bond (polyene or conjugated compound). The increased wavelength of the UVs (200-400 nm) absorbed indicates that the number of double bonds also increased (Hamilton-Miller, 1973; Mohammed, 2018). Besides, compounds reacting positively with the vanillin indicates the presence of carbonyl functional groups that contain a carbon-oxygen double bond (aldehydes, ketones). Accordingly, these compounds probably are from terpenoids, fatty acids/essential oils, steroids, flavonoids, or phenolic groups. A compound that reacts negatively to ninhydrin shows that it is not a nitrogen compound or its derivative. In contrast, negative to ferric (III) chloride indicates that the compound does not have a phenol functional group (Harborne, 1984; Jork, 1990). In this study, several spots in the TLC profile of ethyl acetate extract reacted positively to 365 nm UV light and vanillin reagent, but all of them reacted negatively to ninhydrin and ferric (III) chloride (as shown in Figure 2).

3.3. 3.3. Active Fraction as Antibacterial Against K. pneumoniae and MRSA

After an bioautography test was conducted on *K. pneumoniae* and MRSA pathogens, it was found that the spot with the smallest R_f (0.14) was the active fraction as antibacterial. The results of the bioautography test will help detect the presence of antibacterial compounds by the formation of an inhibition zone around the active spot as shown in Figure 3.

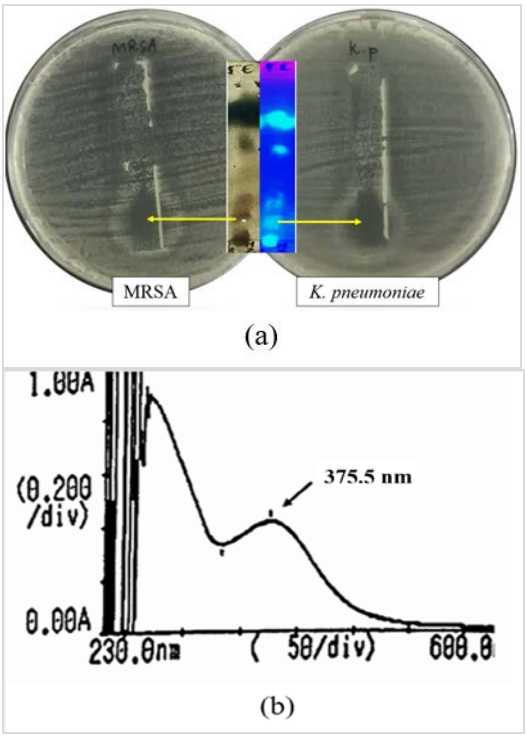

Figure 3. Determination of active fraction as antibacterial **:** (a) Bioautographical results of ethyl acetate extract against MRSA and MDR *K. pneumoniae*, (b) Characteristic of antibacterial active fraction based on spectra patterns towards UV light absorbance

The active fraction was reactive to vanillin and 365 nm UV light on TLC visualization results and was strengthened by the active fraction spectra pattern using a UV-Vis spectrophotometer which has λ 375.5 nm absorption peak (illustrated in Figure 3b). Based on the description of this characteristic, the active fraction is likely thought to have a carbonyl group and contain conjugated double bonds.

3.4. Minimum Inhibitory Concentration of Active Fraction

The cell wall of gram-negative bacteria is thinner, composed of peptidoglycan, 2 layers of phospholipids, and is protected by a lipopolysaccharide capsule. Gram-positive bacteria have thicker walls composed of peptidoglycan and lipoteichoic acid, and 1 layer of phospholipids (Lima *et al*., 2013). Antibacterial activity of the active fraction against MDR *K. pneumoniae* and MRSA pathogens resulted in a similar MIC value, i.e. at 256 µg mL^{-1}. *K. pneumoniae* bacteria are categorized as gram-negative bacteria, while MRSA is gram-positive. Both bacteria are still sensitive to chloramphenicol

antibacterial since their MIC value is less than 8 μg mL^{-1} (CLSI, 2017).

Chloramphenicol is a commercial broad-spectrum antibacterial. Moreover, chloramphenicol can damage important metabolic pathogens by binding the 50S ribosome subunit and blocking essential ribosomal function. The interaction of the nitrobenzyl functional group from chloramphenicol and the bacterial RNA nitrogen base may interfere with the formation of peptides during the process of protein biosynthesis done by bacteria (Kostopoulou *et al*., 2011).

The active fraction resulted from this study had a carbonyl group and also alternating double bonds (conjugation). The aldehyde and ketone carbonyl groups are highly polarized because carbon is less electronegative than oxygen. Carbon contains a partial positive charge (δ^+), while oxygen has a partial negative charge (δ^-). Hence, the carbonyl group can function as a nucleophile and an electrophile. The conjugation of a double bond to the carbonyl group will transmit the electrophilic character of the carbonyl to the beta-carbon of the other double bonds, or popularly called charge delocalization (Sarker and Nahar, 2007). Charged compounds ions will make them easier to interact with bacterial cell wall so that they can penetrate the cytoplasm membrane.

The mechanism of action of the active fraction as an antibacterial is assumed to be related to its ability to form electrophile sites, i.e. C with δ^+ partial charge which will electrostatically interact with the phospholipid head (PO_4^-) on the surface of the bacterial cell wall. According to Malanovic and Lohner (2016), a positive charge is essential for the initial binding to the surface of the bacterial membrane with a negative charge, which allows it to enter the bacterial cell membrane. Furthermore, these active compounds can affect the metabolic activity of bacterial cells which will eventually cause growth retardation or even death of pathogens.

T. longibrachiatum fungus does not only live in association with sponges in the sea. However, it has also been previously found in soft corals from the water of Panjang Island, Central Java. In addition, its ethyl acetate extract was able to inhibit the growth of MDR-*S. haemolyticus* and produced a 12.2 mm inhibition zone at a concentration of 300 μg $disc^{-1}$ (Sabdaningsih *et al*., 2017). The secondary metabolite from the same fungus has been published by Sperry *et al.* (1998). The ethyl acetate extract of *T. longibrachiatum* is associated with *Haliclona sp.* sponge from Sulawesi water containing an epoxysorbicillinol ($C_{14}H_{16}O_5$), is a member of sorbicillinoids (vertinoids) polyketide compounds. According to Harned and Volp (2011); Meng *et al*. (2016); Salo *et al*. (2016), sorbillinoids are secondary metabolites of hexaketide that undergo cyclization at the carboxylate terminus. Its chemical structure has several double bonds and carbonyl groups. The results of a study from Corral *et al*. (2018) showed that some of these have antibacterial activity, such as sorbicillin ($C_{14}H_{16}O_3$), sorbicillinol ($C_{14}H_{16}O_4$), dihydrosorbicillin ($C_{14}H_{18}O_4$), oxosorbicillinol ($C_{14}H_{16}O_5$), bisvertinol ($C_{28}H_{34}O_8$), and bisvertinolone ($C_{28}H_{32}O_9$). These compounds can inhibit pathogens *Acinetobacter baumannii*, *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*.

4. Conclusion

T. longibrachiatum fungi extracts contain an active fraction that can be developed as an antibacterial against gram-negative pathogens *MDR K. pneumoniae* and gram-positive MRSA. The active fraction is assumed to contain a carbonyl functional group and a conjugated double bond. The mechanism of its antibacterial action is related to the formation of electrophile sites on carbon. Thus, electrostatic interactions occur with negative charges on the cell walls of both gram-positive and gram-negative bacteria making it possible to penetrate the cytoplasmic wall. The active fraction of ethyl acetate extract was antibacterial against pathogens MDR *K. pneumoniae* and MRSA with the same MIC value, i.e. 256 μg mL^{-1}.

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Table 1. MIC value of active fraction and chloramphenicol against MDR *K. pneumoniae* and MRSA pathogens

Tested bacteria	Value of MIC (μg mL^{-1})	
	Active fraction	Chloramphenicol (positive control)
MRSA	256	4
K. pneumoniae	256	4

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Gene Expression of Heat Shock Protein 90 (HSP90AA1 and HSP90AB1) in Thyroid Disorders Patients

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Abstract

The thyroid gland can produce a standard amount of thyroid hormone in response to serum Thyroid Stimulating Hormone (TSH). Hormones of the thyroid gland control the metabolism of cells and their activity speed. Also, they regulate the rate of oxygen consumption. During this study, the gene expression of HSP90AA1 and HSP90AB1 from non-treated hypo and hyperthyroid patients diagnosed by clinical examinations and paraclinical data were compared with a control group. The study included 120 samples collected from patients who suffer from thyroid disorders and 100 samples collected from healthy people as a control group. The age ranged from patients and healthy individuals having (17-79) years. The hormones were measured by ELISA methods; gene expression of HSP90 was measured by RT-PCR using SYBR Green Master after RNA was isolated and converted to cDNA. Significant changes were observed in the level of hormones, in hypothyroidism TSH increased while decreased in hyperthyroidism, whereas triiodothyronine (T3) and Thyroxine (T4) increased in hyperthyroidism and decreased in hypothyroidism. Results of HSP90AA1 gene expression showed the mean of folds was nonsignificantly increased in patients compared with control; while the results of HSP90AB1were high in hyperthyroidism and low in hypothyroidism but these differences were nonsignificant compared with a control group. The expression of HSP90 genes can be used as a risk factor in the diagnosis of thyroid disorders.

Keywords: Folding, TSH, Chaperone, HSP90, HSP90AA1 gene, HSP90AB1gene.

1. Introduction

In humans, the thyroid gland consists of Tow lobes that are lateral and inferior to the anterior part of the larynx and are connected by an isthmus across the larynx to create a U-shaped form, in adults the gland with an average weight of 30 gm (Chiasera, 2013). Thyroxine (T4) and 3,3 ', 5-triiod-L-thyronine (T3) hormones are secreted from the thyroid gland and stored therein. Thyroid hormones control homeostasis of energy, cell proliferation, and metabolism of carbohydrates, fats and proteins (Wallis *et al*., 2010). Among the most common endocrine diseases are thyroid gland disorders. Thus, the study of T3 and T4 thyroid hormones has important biological and medical implications (Demir *et al*., 2020).

Proteins called heat shock proteins (HSPs) are produced when cells exposed to high temperatures become temporarily resistant to subsequent heat shock. HSPs are molecular chaperones that are conserved and grouped by their molecular mass and a high degree of amino acid homology between microbes and humans (Kim and Yenari, 2017). The HSPs range from 15 to 110 kDa in molecular weight. There are groups of binding proteins with a high molecular weight of about: 100, 90, 70, and 60kDa. Also they are classified as small HSPs with a low molecular weight of 12-43 kDa. 80-100 amino acids contain small HSPs (Tkáčová and Angelovičová, 2012).

Heat shock protein 90 (HSP90) is essential for activating several signaling proteins in eukaryotic cells as a molecular chaperone. The structural and biochemical study of HSP90, which promotes the stimulation of HSP90's clientele, has shown a complex mechanism of ATPase-coupled conformation variations and interactions with cochaperone proteins. While recent progress has been made, key aspects of HSP90's ATP-coupled mechanism remain controversial, and therefore the nature of the changes produced by HSP90 in client defense are unknown (Pearl and Prodromou, 2006).

The present study aimed to evaluate changes in HSP90 gene expression and its relationship with thyroid disorders.

2. Methods

2.1. Blood Samples Collection

Ten ml of venous blood were collected from a suitable vein. 3 ml of blood samples were put in a dry EDTA tubes and shaken gently then used for moleculare diagnosis. The residual part of the blood sample was transferred to a glass tube (anticoagulation-free) and allowed to coagulate for serum separation for 5 minutes using a 4000 rpm centrifuge. In a sterile, clean white tube, the extracted serum was collected and stored at -20 °C for thyroid hormones measurement.

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2.2. Measurement of the Hormones levels

Accu-Bind ELISA microwell kit (Monobind Inc, USA) was used for the quantitative determination of Total Triiodothyronine (tT3), and total thyroxine (tT4) concentration in human serum by microplate Enzyme immunoassay. The quantitative immune enzymatic assay of TSH was based on the ELIFA technique by Mini VIDAS according to the manufacturer protocol (Bio Merieux, France).

2.3. Measurement of gene expression of HSP90 by quantitative real-time PCR

Total RNA was isolated from sample blood using Trizol reagent (Invitrogen, USA) according to the protocol of the manufacturer.; The concentration of extracted RNA was estimated by the protocol for quantitating RNA in a single tube using the Quantus fluorometer (Promega, USA). After that, RNA was reverse transcribed for use as a template in the PCR reaction into complementary DNA (cDNA). The reaction to Real Time (RT) was carried out using Accu Power Rocket Script RT PreMix (Bioneer, Korea). This kit is a ready-to-use lyophilized master mix containing all components from the RNA template for first-strand cDNA synthesis.

Table 1. Primer sets for genes analyzed by qRT-PCR.

GAPDH	F	5'-GAAGGTGAAGGTCGGAGTC-3'
GAPDH	R	5'-GAAGATGGTGATGGGATTTC-3'
HSP90AA1	F	5'- TGGAATGACCAAGGCTGACT -3'
HSP90AA1	R	5'- TGAGGACTCCCAAGCGTACT -3'
HSP90AB1	F	5'- TGATGAGGCAGAGGAAGAGAA -3'
HSP90AB1	R	5'- TCTGGTCCAAATAGGCTTGG -3'

The real-time fluorescent quantitative polymerase chain reaction (RT-qPCR) process with GAPDH as a control was used to study the mRNA expression levels of HSP90AA1 and HSP90AB1 in blood samples. The RT-qPCR was carried out according to the SYBR Green Master kit manufacturer's protocol. Table 1 lists the HSP90 gene primers for RT-qPCR. The dissociation curve was evaluated to ensure an apparent amplification peak and validate it. The expression levels of HSP90AA1 and HSP90AB1 were determined using the $2^{-\Delta\Delta}$Ct method and subjected to statistical analysis (Schmittgen and Livak, 2008).

2.4. Statistical

In order to detect the effect of different variables on study parameters, the Statistical Analysis System- SAS (2012) software was used. The least significant difference-LSD test (Analysis of Variation-ANOVA) was used to compare the means significantly.

3. Result

3.1. Levels of Hormones in hypo and hyperthyroidism

The present study showed (Table 2) a significant difference (P≥0.01) in the level of TSH in control, hypothyroidism, and hyperthyroidism. The mean of the TSH level of hyperthyroidism (0.288 µIU/ml) was lower than that of hypothyroidism (7.31 µIU/ml) and control group (2.78 µIU/ml), while the mean of TSH level of hypothyroidism was higher than that of control and hyperthyroidism. Also, the present study showed a significant difference in the level of T3 and T4 in control, hypothyroidism, and hyperthyroidism (P≥0.01). The mean of the level of the hormones in hypothyroidism was lower than that of hyperthyroidism and control group, while the mean of T3 and t4 levels in hyperthyroidism was higher than that of control and hypothyroidism; the T3 means for three groups were 1.71 ng/ml, 0.49 ng/ml, and 3.87ng/ml; and for T4 were 5.84 µg/ml, 1.41 µg/ml, and 10.81 µg/ml respectively.

Table 2. Distribution of the Hormones in Thyroid Disorders Patients

Group	T3 (ng/ml)	T4 (µg/ml)	TSH (µIU/m)
Control	5.84± 0.064 b	1.71± 0.053 b	2.78 ± 0.26 b
Hypothyroidism	1.41± 0.038 c	0.49± 0.062 c	7.31± 0.71 a
Hyperthyroidism	10.81± 0.12 a	3.87± 0.045 a	0.288± 0.021c

Means having the different letters in the same column differed significantly. ** (P≤0.01).

3.2. Gene Expression of HSP90 in hypo and hyperthyroidism

The positive results showed amplification at CT (threshold cycle) value were in range 15.9– 18.48 for housekeeping gene and 19.02- 21.69 for HSP 90AA1 gene and 17.71–20.66 for HSP 90AB1 gene in hyperthyroidism, while in hypothyroidism the CT value was in range 15.74–16.91 for housekeeping gene (Figure 1) and 18.97-20.06 for HSP 90AA1 gene and 17.13–24.67 for HSP 90AB1 gene. The results for the control group showed amplification at CT value was in the range 15.84– 16.34 for housekeeping gene and 18.96-19.94 for HSP 90AA1 gene and 17.67– 22.69 for HSP 90AB1 gene.

The melting temperature (Tm) values obtained for the patients samples of hyperthyroidism, hypothyroidism, and control; the melting temperature was in the range 85.23-85.93, 85.53-85.93, 85.73-86.23ºC respectively for housekeeping gene (Figure 1), HSP90AA1was in the range of 81.54-82.24, 81.64-82.04, 81.74-82.34 ºC respectively, HSP90AB1 was in the range of 79.15-79.84, 79.54-79.84, 79.44- 80.04 respectively.

The results of HSP90 genes expression in Figure (2) showed an increase in gene expression of HSP90AA1 in hypothyroidism and hyperthyroidism, but the increase was nonsignificant differences compared with control group. The mean of folds in hypothyroidism, hyperthyroidism, and control was 0.92±0.34, 0.96±0.33, and 0.82±0.28 respectively. While the results of HSP90AB1 (Figure 2) show a high in gene expression in hyperthyroidism and low gene expression in hypothyroidism, these differences were nonsignificant compared with a control group; the mean of folds was 1.82±0.43 (hypothyroidism), 4.84±2.72 (hyperthyroidism), and 3.81±1.29 (control).

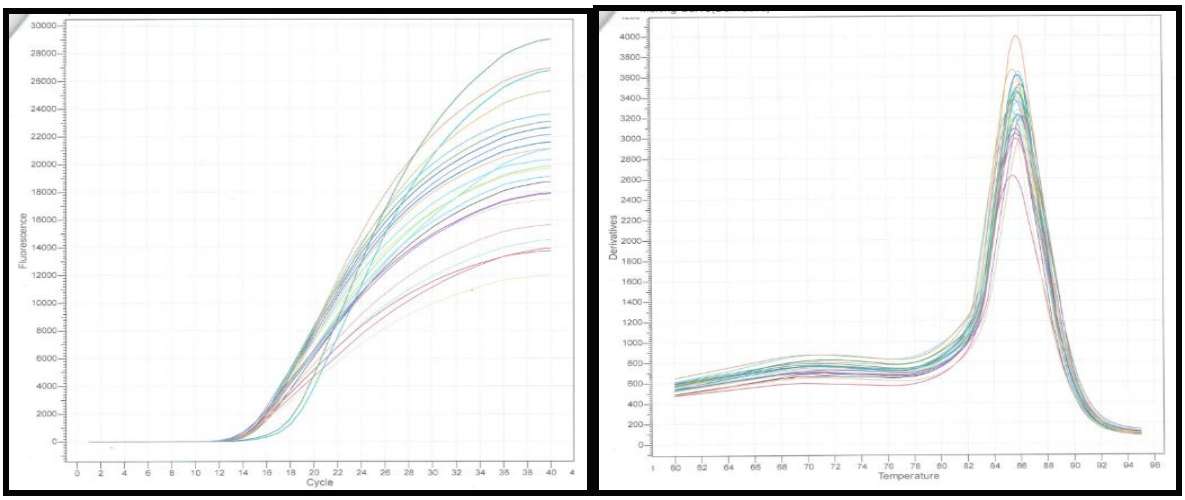

Figure 1. Curve of cycling and melting for housekeeping GAPDH gene

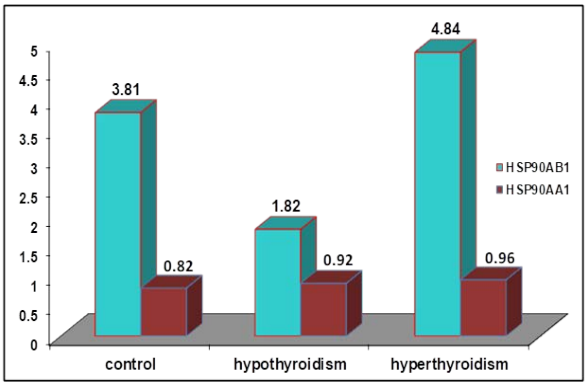

Figure 2. Expression levels of HSP90AA1 and HSP90AB1 gene

4. Discussion

Hyperthyroidism is a clinical condition due to an excessive increase in thyroid hormones, particularly triiodothyronine (T3) and thyroxine (T4). Research by Sugimoto and Mori (2012) showed that in hypothyroidism, TSH levels are raised, owing to the lack of a suppressive action of the T3 and T4. The most common cause of hyperthyroidism is toxic goiter or Graves' disease (Gilles *et al.*, 2008). The hypophysis tells the thyroid how much hormone is needed to produce. It will not be ready to give the thyroid the correct instructions if the pituitary is damaged by injury, a tumor, radiation, or surgery, and so the thyroid may stop producing enough hormones (Dunn and Turner, 2016). Iodine deficiency is the most common cause of hypothyroidism worldwide; and it is the most prevalent cause of hypothyroidism; and too much iodine can also cause or exacerbate hyperthyroidism (Al Hadid *et al.*, 2018).

HSP works on protein transport and assembly, and proper folding of peptide chains, thus playing a crucial role in protecting cells, and ultimately affecting cell survival (Chen *et al.*, 2018), and much research in recent years has demonstrated that HSP is related to cell apoptosis (Zhang *et al.*, 2017). HSP90 exists in the thyroid follicular epithelial cells and follicular cavities (Calderwood, 2018; Yan *et al.*, 2019).

HSP90 is a molecular chaperone that interacts with client proteins (Hertlein *et al.*, 2010), thereby preventing their degradation. For example, because of the stress response to hypoxic, acidic, and nutrient-deprived hostile microenvironment characteristics, expression of HSP90 is increasing in many cancers and correlates with poor prognosis (Bagatell and Whitesell, 2004; Pick *et al.*, 2007). HSP90 levels increase in stressed cells, and it is unclear whether cell stress is necessary for the efficacy of HSP90 inhibitors; and Yu-bao *et al.*, (2014) suggest that HSP90 expression is relative to the disease.

5. Conclusion

The present work illustrates that the expression of the two HSP90 genes changes in both hypothyroidism and hyperthyroidism. Also, the HSP90AA1 gene and HSP90AB1 gene correlate with TSH and Thyroid hormones; thus these genes can be used as risk factors in the diagnosis of thyroid disorders.

Authors' Contributions

ASM, MMA, and NAM contributed to the study design and analyzed data. All authors contributed to the manuscript drafting and revising and approved the final submission.

Competing interests

The authors declare that they have no competing interests associated with this article.

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Sinensetin Contents of Purple and White Purple Variety of *Orthosiphon aristatus* (Blume) Miq

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ABSTRACT:

Context: The cat's whiskers (*Orthosiphon aristatus* (Blume) Miq) plant has been widely used for traditional medicine both in preventive measures and companion therapy. The color of the *O. aristatus* flower is divided into purple, white-purple, and white varieties. To ensure the quality of the *O. aristatus* it is necessary to make standardization efforts. Specific standardization parameters are the identification and determination of marker compound levels. Sinensetin is a marker compound in *O. aristatus*. The research aimed to analyze qualitatively and quantitatively the content of sinensetin in the acetone extract of the purple and white-purple varieties of *O. aristatus* Extraction was carried out using maceration, which began with determining the ratio of the number of leaves and stem simplicia of two varieties of *O. aristatus* and acetone solvent. Sinensetin compound identification was carried out on acetone extract and subfraction. Determination of the levels of sinensetin in acetone extract was carried out by thin-layer densitometry chromatography (TLC-Densitometry). Based on the optimization results using TLC, the weight ratio of the simplicia and the amount of solvent is 1 g of simplicia in 15 mL of solvent. Sinensetin compounds were detected in the leaves' acetone extract and stem of two varieties of *O. aristatus*. The highest sinensetin content was in the acetone extract of purple variety leaves with levels of 0.51% w/w. The acetone extract of the purple variety leaves was further separated by vacuum liquid chromatography and preparative thin-layer chromatography. The results showed a sinensetin compound in the acetone sub-fraction. This study's results can be the basis for the specific characterization of *O. aristatus* especially those grown in Indonesia, to ensure product quality consistency from traditional medicinal plants.

Keywords: *O. aristatus,* purple and white-purple varieties, standardization, sinensetin, qualitative and quantitative analysis.

1. Introduction

O. aristatus is one of the medicinal plants which, based on the results of the research, has various pharmacological activities, including antiviral (Ripim et al., 2018; Faramayuda et al., 2021[a]), prevention and treatment of cancer (Pauzi et al., 2018 rheumatoid treatment and osteoarthritis arthritis (Adawiyah et al., 2018), treating cardiovascular disorders (Abraika et al., 2012), Anti – epilepsy (Kar et al., 2012), enhancing memory (George et al., 2015), antioxidants (Alshawsh et al., 2012; antidiabetic (Mohamed et al., 2011; Mohamed et al., 2010), antiobesity (Yam et al., 2009), treatment of overcome gastric disorders (Yuniarto, et al., 2017). Some studies also report that *O. aristatus* have passed clinical trials (Adnyana et al., 2013; Premgamone et al., 2001). Safety testing of *O. aristatus* extracts in male rats that all animals survived and showed no signs of toxicity. (Muhammad Husin et al., 2001).

Some of the active secondary metabolites of *O. aristatus* are sinensetin, eupatorin and rosmarinate acid (Faramayuda et al., 2021[b]),, and danshensu (Nuengchamnong et al., 2011). *O. aristatus* that grow in Indonesia are classified into three varieties, namely purple, intermediate (white-purple), and white (Faramayuda et al., 2021[c]). The difference between the three varieties can be seen from the morphology of the flower (Faramayuda et al., 2021[d]).

Sinensetin is included in the class of flavonoid compounds, and if they are classified, more specifically are polymethoxy compounds produced by secretory tissue and stored inside or outside the oil glands in plants. Flavone polymethoxy compounds have several pharmacological activities and are part of the plant chemical defense mechanism (Berim and Gang, 2016). The results of a study conducted by Hossain and Ismail in 2016 reported that the level of sinensetin in acetone: water (70:30) extract was 0.32% higher than other solvents (Hossain and Ismail, 2016).

The *O. aristatus* plant has been widely used as raw material for traditional medicine, especially in Indonesia, but many people and industry practitioners use this plant without paying attention to the varieties used. According to Faramayuda, in 2021[b], the methanol extract of the purple variety *O. aristatus* had higher levels of sinensetin

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compared to other varieties. Qualitative and quantitative analysis of sinensetin compounds from acetone extract of two varieties of *O. aristatus* that grow in Indonesia has never been reported. This research can be a reference basis for the specific standardization parameters of the acetone extract of two varieties of *O. aristatus*. Standardization of raw materials is necessary to ensure consistency in the quality of traditional medicinal products.

2. Material and Methods

2.1. Chemicals and reagents:

The chemicals used have the quality of use analysis (pro analysis, p.a), sinensetin (Sigma®), ethyl acetate (Merck®), chloroform (Merck®), acetone (Merck®), silica gel plate pre-coating 60 F254 (Merck®), aqua dest, and silica gel 60 GF254 (Merck®).

Instrumentation:

Glass tools are commonly used in laboratories, macerators, analytical scales (Shimadzu), chambers, ovens (memert), rotary evaporators (Heidolph), vacuum liquid chromatography, and TLC-Densitometry (Camag, Switzerland).

Collection O. aristatus plants

The purple and white-purple varieties of leaves and stems of *O. aristatus* were collected from the Manoko experimental garden, Lembang, West Bandung, Indonesia. The plants were identified at the School of Life Science and Technology, Bandung Institute of Technology. With letter number 6115 / I1.CO2.2 / PL / 2019 .

2.2. Preparation of Extraction

The two varieties of leaves and stems of *O. aristatus* were weighed 1 g each and then added 5 mL of acetone, then let stand for 24 hours. The second day the TLC filtrate I profile was observed, and then the residue was added again with 2.5 mL of acetone. On the third day, the TLC filtrate II profile was observed, and then the residue was added again with 2.5 mL of acetone. Day Four was observed profile of TLC filtrate III. The mobile phase used in TLC observation is chloroform: ethyl acetate 60:40 (Figure. 1).

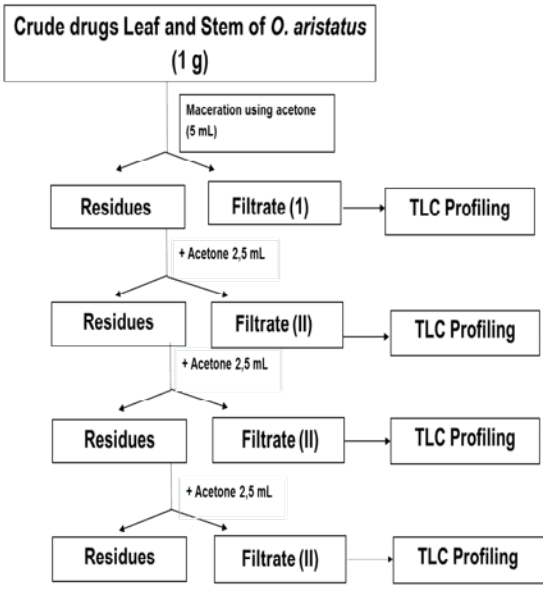

Figure 1. Extraction optimization scheme with maceration

2.3. Extraction of Plant Material

The solvents used to extract plant material are acetone. 100 g of leaves and stems of *O. aristatus* purple variety transferred to macerators, and each added with 1,5 L acetone solvent then macerated for 24 hours. The filtrate was collected, then evaporated using a *rotary evaporator* and concentrated using water baths to form a thick extract.

2.4. Quantitative Analysis

Preparation of standard and sample solutions

Stock solutions of 1 mg / mL of sinensetin in methanol were prepared. The stock solution was diluted with methanol at five concentrations ranging from 60 to 100 µg/mL to create standard solutions. Extracts of acetone from two types of *O. aristatus* were prepared by dissolving 15 mg of each extract in 1 mL of methanol for 45 min as a sample solution.

Instrumentation

Monitoring was carried out in the CAMAG analyzer at a wavelength of 365 nm. Data analysis was carried out using the WinCATS app.

Chromatographic conditions

Standards and samples were applied to a TLC plate with a height of 10 cm and a length of 20 cm. Application volume of 5 mL was performed at a distance of 1 cm from the TLC plate's edge. Toluene mobile phase: ethyl acetate: formic acid: water (3: 3: 1: 0,2) was pre-saturated in the chamber. Observed at a wavelength of 365 nm with densitometry.

2.5. Further separation of the purple variety of O. aristatus acetone extract

Vacuum Liquid Chromatography

The acetone extract was separated by vacuum liquid chromatography using silica gel 60 stationary phases, and 5 g extract was used. The mobile phase used is chloroform: ethyl acetate (60:40) with an isocratic system. The extract was crushed with a small amount of silica gel 60. It then placed it at the top of the column, elution with a mobile phase. Subfraction solutions coming out of the column are collected by volume, which is held every 60 mL. A thin layer chromatography profile was monitored in UV light of 366 nm to the subfraction.

TLC-Preparative and sinensetin Identification

The stationary phase is prepared by mixing 25 grams of silica gel 60 F254 with 50 mL aqua dest (1: 2), shaking in Erlenmeyer (± 90 seconds) until homogeneous. The stationary phase is then poured and flattened on a glass plate measuring 20x20 cm, then allowed to stand for 24 hours in the oven at 100ºC for 30-60 minutes. The mobile phase of chloroform and ethyl acetate 60:40 (Hossain and Ismail, 2016) is put into the chamber and left for 60 minutes, then the subfraction specks on the silica plate. In the elution stage, a plate containing the sample is put into the chamber. The band formed is scraped and dissolved by the mobile phase. The filtrate was evaporated, and monitored the purity of the isolates with TLC.

Analysis of data

Determine the levels of the sinensetin using TLC-Densitometry. All test samples were prepared in three replications. The data are expressed as a mean ± SD. Data processing was carried out by one-way ANOVA, followed

by a multiple-range test by Duncan using SPSS 22 software. P values <0.05 have been considered statistically significant.

3. Result

3.1. Optimazation extraction

Based on the results of the determination of two plant samples identified *O. aristatus* purple flowers and *O.aristatus* white-purple flowers. The optimization of sinensetin extraction with monitoring parameters of the TLC profile showed that on the fourth day, fluorescence from sinensetin was fading on acetone extract of leaves and stems of two varieties of *O. aristatus*. The dry matter ratio used with solvents to maximize sinensetin withdrawal is 1 g: 15 mL (figure 2).

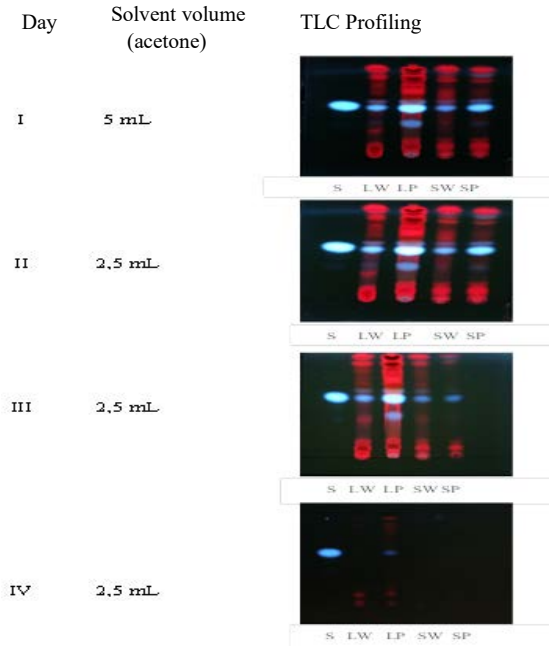

Figure 2. Extraction optimization with TLC profile parameter. Stationary phase silica gel 60 F254, mobile phase chloroform: ethyl acetate (60:40), Rf sinensetin : 0.61. S: sinensetin, LW: leaf extract white-purple., LP: leaf extract purple, SW: stem extract white-purple, SP: stem extract purple.

3.2. Quantitative Analysis

The analysis of sinensetin levels in the acetone extract of the *O. aristatus* leaves and stems used densitometric instruments. The regression equation of the variation in sinensetin's standard concentration is y = 86.872x - 4438.5 with an r^2 value of 0.9954. Three repetitions were carried out for each sample, and the sinensetin compound was detected at Rf 0.57 (figure 3).

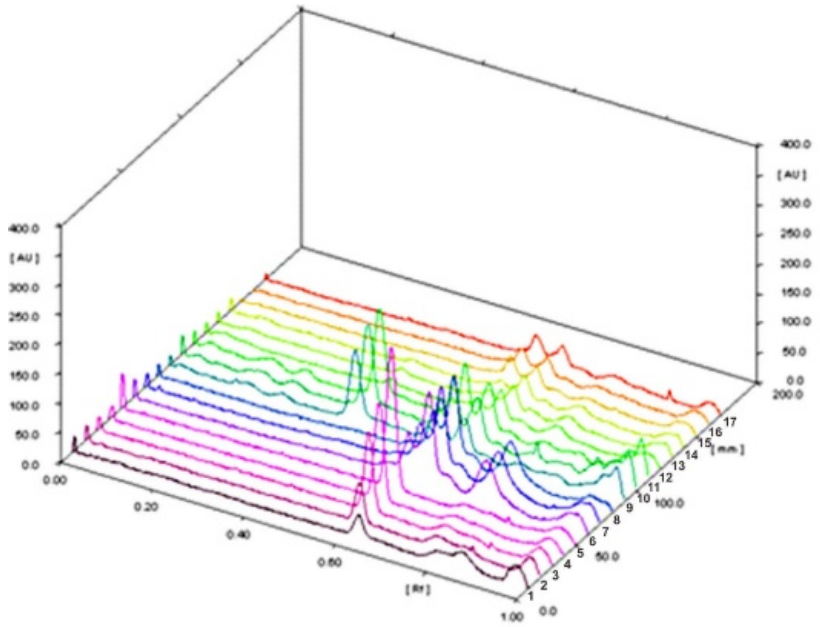

Figure 3. 3D-TLC chromatogram of the acetone extract of two varieties *O. aristatus* and the standard sinensetin. Track 1: sinensetin 60 µg / mL, 2: sinensetin 70 µg / mL, 3: sinensetin 80 µg / mL, 4: sinensetin 90 µg / mL, 5: sinensetin 100 µg / mL, 6-8: purple variety leaves acetone extract (3 replications), 9-11: white-purple variety leaves acetone extract (3 times replication), 12-14: purple variety stem acetone extract (3 replications), 15-17: white-purple variety stem acetone extract (3 times replication).

Table 1. Levels Determination of sinensetin acetone extract of two varieties *O. aristatus* with TLC-densitometry

Sample	Sinensetin (% w / w) ± SD (n = 3)
purple variety leaves acetone extract	0.51 ± 0.01[a]
white-purple variety leaves acetone extract	0.48 ± 0.01[a]
purple variety stem acetone extract	0.43± 0.01[b]
white-purple variety stem acetone extract	0.39 ± 0.02[b]

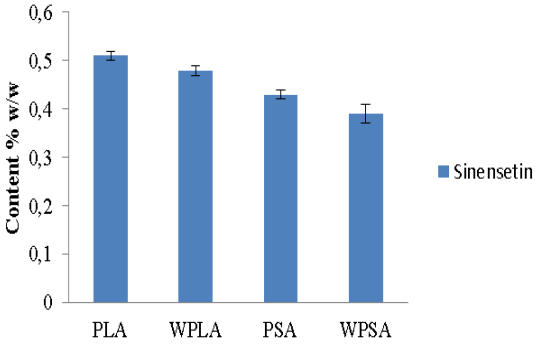

Figure 4. Comparison sinensetin levels of acetone extracts of two varieties of *O. aristatus*. PLA = purple variety leaves acetone extract, WPLA = white-purple variety leaves acetone extract, PSA = purple variety stem acetone extract, WPSA = white-purple variety stem acetone extract

3.3. Further separation of the purple variety of O. aristatus acetone extract

In the separation stage using vacuum liquid chromatography using a mobile phase of chloroform: ethyl acetate 60: 40 with an isocratic system, eight subfractions were obtained. Sub-faction 1-5 contains a sinensetin compound (Rf 0.45), but what is continued to the separation stage is sub-faction 5 (SF5) because there is not too much chlorophyll, so it is easier to isolate (Figure 5). Further separation of SF5 was using preparative thin-layer chromatography (PTLC) with the mobile phase of chloroform: ethyl acetate 60: 40 obtained two bands. The second band is separated by scraping and then dissolved in a mobile phase to filter further and identify the filtrate (figure 6).

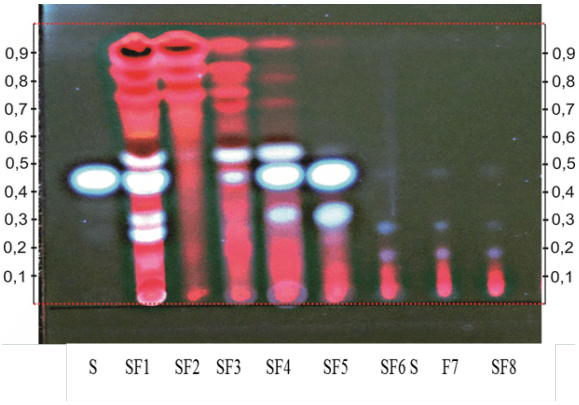

Figure 5. TLC Profile Sub Fraction 1-8 of *O. aristatus*. Stationary phase silica gel 60 F254, mobil phase = chloroform : ethyl acetate 60 : 40. S: Sinensetin, SF1 = sub-faction 1, SF2 = sub-faction 2, SF3 = sub-faction 3, SF4 = sub-faction 4, SF5 = sub-faction 5, SF6 = sub-faction 6, SF7 = sub-faction 7, SF8 = sub-faction 8.

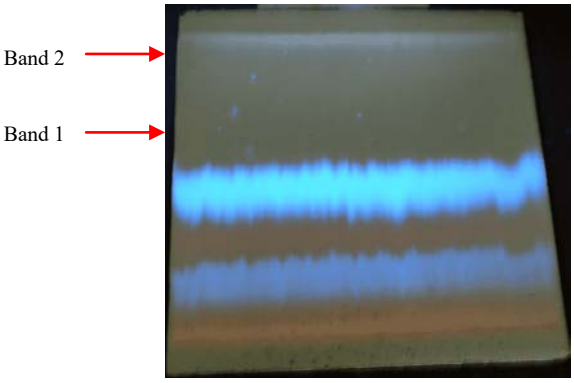

Figure 6. TLC Preparative Profile of subfraction 5. Stationary phase silica gel 60 F254, mobile phase chloroform: ethyl acetate 60: 40

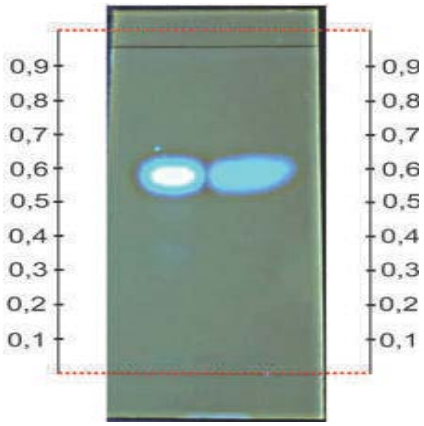

Figure 7. TLC Profile of isolate. Stationary phase silica gel 60 F254; mobile phase chloroform: ethyl acetate 60: 40. S: Sinensertin and I: Isolate

Band 2 filtrate was identified using TLC and Sinensetin standard; the results showed the same Rf between isolate and standard (figure 7).

4. Discussion

4.1. Optimazation extraction

From monitoring the TLC profile, the fluorescence of the sinensetin purple variety is brighter than the white-purple variety; this shows that the purple variety level is higher than that of the white-purple variety. Earlier research reports that the purple variety of sinensetin levels were higher than white varieties (Lee, 2004). According to research conducted by Faramayuda in 2021, it was reported that the population of purple varieties of cat whiskers is decreasing (Faramayuda et al., 2021[e]). Therefore, it is necessary to propagate plants, one of which is the plant tissue culture method. Several studies have reported that the propagation technique with modified in vitro culture has succeeded in producing seeds of Phalaenopsis amabilis (L.) Blume (Mose et al., 2020), Artemisia herba-alba (Shibli et al., 2017), Chrysanthemum morifolium (Shatnawi et al. al., 2010).

4.2. Quantitative Analysis

The sinensetin compound's content in the acetone extract of the purple variety *O. aristatus* was 0.51% w/w, while the white-purple variety was 0.48% w/w. Statistically, there was no significant difference. Two varieties of sinensetin cat whiskers were detected with

levels of 0.43% w / w on the purple variety and 0.39% w/w on the white-purple variety (table 1; figure 4). Sinensetin levels in purple varieties were more significant than those in white-purple varieties. This result was in line with those reported by Febjlasmi in 2018, where the levels of sinensetin from the purple variety *O. aristatus* were greater than the white and white-purple varieties. The levels of sinensetin in the acetone extract of the purple variety cat whiskers were more significant than that reported by previous researchers, where the sinensetin content of the acetone: water extract (70:30) cat whiskers was 0.32% w / w and methanol: water (1; 1) 0.15. % w / w (Hossain et al., 2016). Cai (2018) explains that the levels of sinensetin in the 50% ethanol extract of cat whiskers are 0.27% w / w and in the stem is 0.01% w / w. The average concentration of the sinensetin compound in the purple variety cat whiskers extract that grows at 18 locations in Indonesia is 0.043% w / w. Methanol extract: water (50: 50) of cat whiskers that grow in Malaysia is 0.0057% w / w (Guo et al., 2019). Sinensetin content of 70% ethanol extract of purple variety cat whiskers is 0.0182% w / w (Batubara et al, 2020). Sinensetin, which is lipophilic, is more stable in low polar solvents such as isopropanol, chloroform, and acetone (pang and gimbung, 2014). Based on the chemical structure, sinensetin is a polymethoxy (pentamethoxyflavone) flavone group, the methoxy group substitution at positions 5, 6, 7, 3 '4' (Han Jie et al., 2021). The presence of this methoxy group makes sinensetin less polar.

4.3. Further separation of the purple variety of O. aristatus acetone extract

In the Further separation, the plant used is purple variety leaves. The acetone solvent selection in sinensetin isolation was based on Hossain's research, and Ismail in 2016 reported that the acetone extract: water has higher sinensetin levels than other extracts (Hossain and Ismail, 2016). The yield of acetone extract is 18.5% w / w. The isolates suspected of being sinensetin were obtained as much as 1.8 mg from 100 gr of simplicia *O. aristatus* purple varieties. The optimization of sinensetin separation by VLC will be better if the elution were performed by gradient elution with n-hexane-ethyl acetate. In this research the solvent used for VLC was too polar. Several studies on the isolation and identification of sinensetin have been reported, including sinensetin obtained from the ethyl acetate fraction of *O. aristatus,* which was separated using column chromatography and then eluted with 100% hexane, followed by up to 100% hexane: ethyl acetate and up to 100% ethyl acetate: methanol (Samidurai et al., 2019). The methanol extract of the leaves was extracted with-hexane, chloroform, ethyl acetate, and butanol. The chloroform fraction was purified, and six compounds were obtained, one of which is sinensetin (Hossain and Mizanur Rahman, 2015). Two methoxy flavonoids were isolated and identified from the leaves of *Orthosiphon stamineus*, Benth, which are known as sinensetin and flavones 5,7,8,4' tetra methoxy, extraction with Soxhlet using ethyl acetate as a solvent followed by liquid-liquid extraction with $CuCl_2$ and NaOH to reduce the effect chlorophyll content in the isolation process (Febriani et al.,2017). The HPLC method was developed for the separation and determination of three methoxylated flavones: sinensetin, eupatorium, and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone in *Orthosiphon stamineus*. All compounds were separated using reverse phase C18, Lichrosorb column with methanol-water mobile phase -tetrahydrofuran 45: 50: 5 v / v (Akowuah, et al., 2004). This study provides additional information regarding the composition of the number of simplicia and solvents, which can then be developed for the basis of production or optimization of the isolation of sinensetin from *O. aristatus*. Several previous studies reported attempts to isolate sinensetin, including 4 kg of simplicia of the cat's whiskers producing 75 mg of sinensetin (Tezuka et al., 2000). Isolation of sinensetin from 500 g of *O. aristatus* using an LH-20 Sephadex column resulted in an isolate of 3.03 mg (Yuliana et al., 2009). Hossain and Mizanur Rahman (2015) reported that from 1 kg of plant material, *O. aristatus* produced 2.6 mg of sinensetin isolate.

5. Conclusion

The acetone extract of the leaves and stem of the purple variety *O. aristatus* had higher sinensetin levels than the white-purple variety.

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Conflict of Interest

The authors declare no conflict of interest.

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Lactobacillus reuteri and *Lactobacillus salivarius*, Two Prevalent Vaginal Species Isolated from Healthy Women in Western Algeria.

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Abstract

Background & objectives: *Lactobacillus* species or their metabolites (such as lactic acid or bacteriocin) are known to protect the vagina from urogenital infections and to maintain vaginal hygiene. The goal of the current study was to identify lactobacilli species for a possible probiotic and postbiotic perspective. In Mostaganem city (Algeria), an effective molecular detective technique for lactobacilli in the vagina of women is seriously lacking. Therefore, our current studies promise to contribute to molecular diagnostic.

Materials and Methods: 37 samples of vaginal lactobacilli from 32 healthy women were isolated. By using polymerase chain reaction (PCR), strains were identified. For identification, genus specific, group specific and species-specific PCR protocols were implemented.

Results: All isolates were confirmed as spp. *Lactobacillus.* All 37 isolates belonged to Group IV of the *Lactobacillus* genus and were further divided into two *L. reuteri* and *L. salivarius* species.

Result interpretation & conclusion: In this study, the prevalent species in vagina were *L. reuteri* (70, 27%), followed by *L. salivarius* (29,73%). The probiotic potential of *L. salivarius* and *L. reuteri* from vaginal origin has not been fully exploited yet but studies to date have shown that these two species may be reliable candidates for use as new probiotics or as 'postbiotic' metabolites.

Keywords: Vaginal lactobacilli; Multiplex PCR; *Lactobacillus reuteri*; *Lactobacillus salivarius*; Probiotics; Postbiotics.

1. Introduction

The presence of beneficial bacteria which product lactic-acid- in the vaginal area, particularly from the *Lactobacillus* genus, is what defines vaginal eubiosis. *Lactobacillus* spp. can produce vaginal eubiosis by killing healthy and pathogenic bacteria by lactic acid, either natively or as probiotics. Other antimicrobial factors can also be released, such as bacteriocins (Aroutcheva et al., 2001; Selle and Klaenhammer, 2013).

Recent studies have shown that lactic acid **(**O'Hanlon, 2011; Gong et al., 2014) is the key antimicrobial factor produced by lactobacilli. It was proposed by Gil et al. (2010) that *L. salivarius* was the top source of lactic acid. The benefits of *L. reuteri* have also been enumerated by various reports as a probiotic (Indrio et al., 2008; Spinler et al., 2008; Hou et al., 2015). *L. reuteri* in both bacteriocidal and bacteriolytic forms, produced an antimicrobial agent (bacteriocin) which was active against sensitive cells (Kawai et al., 2001). Bacteriocins generation by *L. salivarius* has been also identified (Barrett et al., 2007; Strahinic et al., 2007; Busarcevic et al., 2008).

A clearer understanding of each population's natural vaginal biota seems important in order to suggest improved probiotics. Some studies have analyzed vaginal microbiota and reported numerous findings from different populations (Vasquez et al., 2002; Zhou et al., 2007). Some studies have confirmed that *L. crispatus*, *L. gasseri, L. jensenii*, and *L. iners* were predominated in healthy vaginal microbiota **(**Ravel et al., 2011), but different findings were documented in other studies (Garg et al., 2009; Damelin et al., 2011).

Within the *L. acidophilus* the closely related species it is really challenging. and often hard to distinguish *acidophilus* complexes by phenotypic techniques (Klein et al.,1998). Furthermore, so many of the distinctions of vaginal *Lactobacillus* observed in numerous researches may be due to the lack of effective detection methods **(**Redondo-Lopez et al., 1990; Zhong et al., 1998). Accurate molecular biological methods are therefore required to identify the presence or type of vaginal

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Lactobacillus, not just for infection therapy, but also to restore the natural microbiota in a variety of conditions.

Multiplex PCR methods have also been used for the simultaneous identification and discriminating of a large number of bacteria. Multiplex PCR was performed utilizing 16S rRNA or 16S-23S rRNA intergenic spacer region (ISR) primers based on sequences unique to each species encompassing the 16S rRNA or 23S rRNA flanking region (Schleifer et al., 1995).

Probiotics, particularly *Lactobacillus* species, have gained prominence in recent years. There are few studies on *L. salivarius* and *L. reuteri* from the human vagina and their use as a probiotic or its 'postbiotic' metabolites to prevent or treat vaginal disorders, opening the door to this important public health issue.

2. Materials and methods

2.1. Specimen collection

Vaginal isolates were collected by a midwife from 32 volunteer women working in Oued El Kheir public health unit in Mostaganem city (Algeria), who were premenopausal, non-menstruating between the ages of 18 and 45. For these experimental purposes, volunteer consent was obtained. With cotton swabs, vaginal samples were taken aseptically.

2.2. Lactobacillus strains isolation from vaginal swabs

Once vaginal swab samples were collected, they were sent to the bacteriology laboratory of Aïn-Tedeles Public Hospital, in Mostaganem city (Algeria). The swabs were inoculated into 5 ml MRS (de Man, Rogosa, and Sharpe) broth and allowed to enrich for 24 hours at 37 °C. MRS plates were streaked with the enhanced bacterial suspension and incubated at 37 °C for 48 hours under anaerobic conditions (Gordana et al., 2011).

2.3. Phenotypic characterization

All isolates were first examined for colony shape, Gram stain, catalase, and oxidase activity. These studies were carried out in the city of Mostaganem's Microbiology and Plant Biology laboratory (Algeria). All isolates that were gram-positive and catalase-negative, were stored in 30 percent glycerol at -80 °C.

2.4. Molecular Diagnosis

Genotypic identification of the lactobacilli isolates was carried out at Microbiology Laboratory in the Faculty of Veterinary Medicine at Mustafa Kamel University, Antakya city (Turkey).

2.5. Genomic DNA extraction:

Isolates were streaked on MRS agar for nucleic acid isolation. Only one colony from each type of strain resuspended in 500 µl of sterile PBS (phosphate buffer saline pH: 7.2) after an overnight incubation at 37°C in microaerophilic conditions. Bacterial cells were collected by centrifugation at 3000 x g for 10 minutes, then resuspended in 350 µl TE buffer [10 mM tris chloride, 1 mM EDTA (pH8.0)] with 20 mg of lysozyme (Sigma, USA) per ml and incubated at 37°C for 60 minutes. Every 15 minutes, each tube was vortexed. Then 350 µl 10 percent SDS was added, along with 100 g proteinase-K (Vivantis Technologies, Malaysia) per ml, then incubated for 60 minutes at 37°C. According to Sambrook and Russell, (2001) the phenol/chloroform extraction technique was employed for nucleic acid extraction. The precipitate of DNA was diluted in 100 µl of TE buffer [10 mM Tris chloride-1 mM EDTA (pH 8.0)], and kept at -20°C till use.

2.6. PCR analysis

2.6.1. Genus Specific PCR:

Amplification using the genus-specific primer LbLMA-rev (5' CTC AAA ACT AAA CAA AGT TTC3') and the universal primer R16-1 (5' CTT GTA CAC ACC GCC CGT TCA3') was used to identify each isolate at the genus level. Each PCR was carried out in a 25 µl volume of the reaction comprising 2 µl of DNA that has been extracted, 2.5 µl of the Taq buffer (10X, Vivantis, Malaysia), 200 µM of each of the deoxynucleotide triphosphates (dNTP), 20 µM of each of the forward and reverse primers, and 1 U of the Taq DNA polymerase (Vivantis, Malaysia).

The reaction protocol was set at initial denaturation at 94°C for 5 minutes was followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C, and extension at 72°C for 30 seconds and 7 minutes (Dubernet and Desmasures, 2002)**.**

2.6.2. Group Specific Multiplex PCR

After verifying the genus and to establish which group an isolate belonged to, multiplex PCR-G was employed using a primer mixture that contains equimolar amounts of four forward primers, Ldel 7(5'ACAGATGGATGGAGAGCAGA3'), LU-1'(5'ATTGTAGAGCGACCGAGAAG3'), LU 3'(5'AAACCGAGAACACCGCGTT3'), LU-5' (5'CTAGCGGGTGCACTTTGTT3') and 1 common reverse primer Lac-2 (5'CCTCTTCGCTCGCCGCTACT3') as per Song *et al.* (2000).

Each PCR was carried out in a 25 µl reaction volume containing 2 µl of extracted DNA, 2.5 µl of the Taq buffer, 200 µM of each dNTP, 20 µM of each of reverse and forward primers, and 1 U of the Taq DNA polymerase. for group specific PCR, a program of denaturation at 95°C for 1 minute, then 35 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 2 minutes, and extension at 72°C for 30 seconds and 7 minutes thereafter, was employed.

2.6.3. Species Specific Multiplex PCR

After group detection, a species-specific multiplex PCR was used according to Song *et al*. (2000), using the primers shown in **table 1**. Each PCR was carried out in a 25 µl the volume of the reaction containing 2 µl of the DNA that has been extracted, 2.5 µl of the Taq buffer, 200 µM of each dNTP, 20 µM of each of the forward and reverse primers, and 1 U of the Taq DNA polymerase. PCR program for species specific multiplex PCR was employed at denaturation at 95°C for 3 minutes then, 35 cycles of denaturation for 20 second at 95°C, annealing for 60 second at 60°C and extension for 60 sec at 72^{o}C and thereafter 7 minutes for final extension.

Table 1: Species that have been identified by PCR

Program	Primer (5'-3' sequence)	Species	Amplicon (bp)
	Lsal-1	*L. salivarius*	411
	AATCGCTAAACTCATAACCT		
	Lsal-2		
	CACTCTCTTTGGCTAATCTT		
	Lreu-1		
	CAGACAATCTTTGATTGTTTAG	*L. reuteri*	303
	Lreu-4		
PCR- IV	GCTTGTTGGTTTGGGCTCTTC		
	Lpla-3	*L. plantarum*	
	ATTCATAGTCTAGTTGGAGGT		
	Lpla-2		248
	CCTGAACTGAGAGAATTTGA		
	Lfer-3		
	ACTAACTTGACTGATCTACGA	*L. fermentum*	192
	Lfer-4		
	TTCACTGCTCAAGTAATCATC		

2.7. Agarose Gel Electrophoresis and Imaging

Electrophoresis of aliquots of the amplified products in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2) on 2% agarose gels was performed. Ethidium bromide was used to stain the gels, which were then viewed under UV light. As a molecular mass marker, a 100-bp polymer was employed (Vivantis).

3. Results and Discussion

3.1. Molecular identification

The results of genus specific PCR are shown in **fig.1,** which indicates that all vaginal isolates tested gave an amplicon of about 200 bp long. In the results of group specific PCR, all isolates detected belong to group IV with amplicons of 350 bp and in the species-specific PCR detection two different species were identified. The amplification products of group and species-specific PCR were shown in **fig.2**.

In the analyses of species, 26 (70, 27%) of isolates were found to be *L. reuteri* (303 bp) and 11 (29, 73%) *L. salivarius* (411 bp). These 2 species of *Lactobacillus* belong to group IV **(fig.2).**

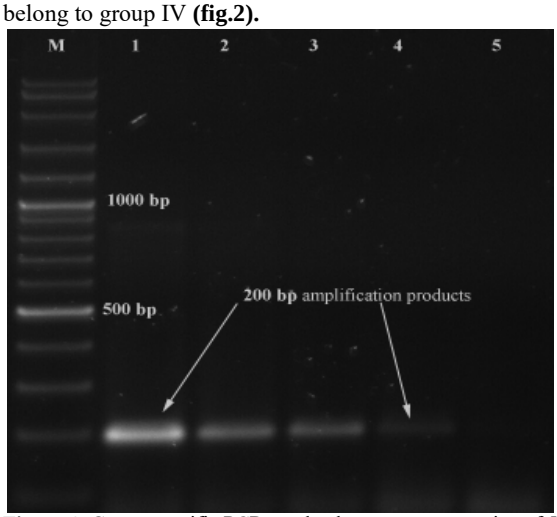

Figure 1. Genus-specific PCR results that are representative of 5 isolates on 2% agarose gel.

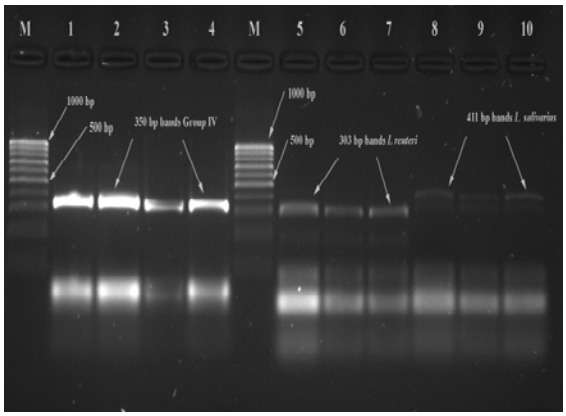

Figure 2 Agarose gel electrophoresis of PCR products from multiplex PCR-G and species-specific multiplex PCR.

It was necessary to first identify the *Lactobacillus* species that colonize women in this region before administering exogenous *Lactobacillus* or their metabolites to preserve vaginal health in west Algerian women.

Vaginal isolates were characterized in our sample base d on cell morphology and physiological and biochemical studies.

Our finding reveals difficulties in separating these isolates on the basis of tests for the fermentation of carbohydrates, which are also supported by Chagnaud et al. (2001) who also reported the reference *L. salivarius* UCC43321 strain as *L. Paracasei* with this technique. Thus, phenotypically characterized strains, even those with a good level of identification, could be misidentified. According to Song et al. (1999), API tests typically identify *L. acidophilus* isolates as belonging to the species *L. crispatus.*

Molecular techniques, such as PCR, can reduce observer-dependent errors and offer more comprehensive and precise findings than biochemical approaches (Yan et al., 2009). On the basis of Gram positivity and catalase negativity, genomic DNA was amplified from 37 vaginal isolates of 32 women cultured on MRS medium and identified as *Lactobacillus*. By genus-specific PCR **(Fig 1),** all of the isolates examined had an amplicon of around 200 bp, indicating that they all belonged to the *Lactobacillus* genus (Garg et al., 2009).

The group-specific PCR utilized a combination of one reverse primer and forward primers to distinguish *Lactobacillus* into classes I, II, III, and IV. A total of 100% of the isolates with a 350 bp amplicon were found to belong to group IV **(Fig 2)**.

In a study conducted by Garg et al. (2009), 80 vaginal *Lactobacillus* isolates using group-specific PCR were examined. They found that 80% of isolates belonged to Group IV, 13.75% to Group II, and 6.25% to women's Group III. There was no isolate belonging to Category I.

All the 37 isolates were analyzed at species level, by species specific primers. These species were identified as *L. reuteri* and *L. salivarius*. These two species which are part of the usual vaginal microbiota were tested for their essential characteristics as possible probiotics (data not shown). Garg et al. (2009) found that *L. salivarius* (16.25%), *L. fermentum* (25%), and *L. reuteri* (32.5%) were the most frequent species in women's vaginal

Lactobacillus species in Delhi, northern India, when they used the same primers, we used.

While the species *L. crispatus* and *L. jensenii* were discovered in Mysore, southern India, and are similar to those identified in American women studied by Pavlova et al (2002), such variations imply that vaginal *Lactobacillus* species can differ across Indian subpopulations. *Lactobacillus crispatus*, *L. gasseri*, and *L.jensenii* are the most widely cultivated vaginal lactobacilli, according to studies from throughout the world (Chaban et al., 2014; Mendes-Soares et al., 2014; Van de Wijgert et al., 2014).

L. acidophilus and *L. fermentum* dominated the vaginal biota of healthy women in the past, *L. brevis*, *L. jensenii*, and *L. casei* are the next species., and other species (Lachlak et al., 1996). It is also worth noting that the quantity of prevalent lactobacilli differs significantly between women from various ethnic groupings. For example, *L. crispatus*, *L. iners, L. gasseri*, and *L. jensenii* were found in 91%, 75%, 67%, and, 43% of healthy Chinese women, respectively (Yan et al., 2009). But the abundance of these lactobacilli in Iranian women was 66.7%, 55%, 29.6% and, 29.6% respectively for the same species (Motevaseli et al., 2013).

We also note major variations in Algeria, with two species, *L. iners* and *L. delbrueckii*, identified in the study conducted by Alioua *et al*. **(**Alioua et al., 2016) on 15 pregnant women in Annaba, eastern Algeria. Although *L. gasseri* and *L. plantarum* were found in Jijel, northeastern Algeria, they were obtained from 60 healthy women (Bouridane et al., 2016). *L. fermentum* was the dominant species identified by Ouarabi *et al*. (2017), followed by *L. plantarum* in 10 healthy women from Bejaia, another region in eastern Algeria.

It is crucial to understand that the presence of certain health-promoting LAB (Lactic Acid Bacteria) genera in the vaginal canal, such as *L. reuteri*, could be a source for inoculation of newborn babies' guts during vaginal delivery for initial colonization of the neonates' colon with these beneficial bacteria (Al-Balawi and Morsy, 2020).

4. Conclusion

This study shows that there are similarities and variations between the vaginal lactobacilli composition found in Algeria and those in environments that are markedly different.

The vaginal biota of Algerian women is little studied., and particularly in the western region, so our research fills a gap in the literature. The results of this study also indicate that *L. reuteri* and *L. salivarius* are promising candidates for probiotics or postbiotics that are usually isolated from healthy Algerian women.

These findings suggest that further research is needed into the microbial variety of women's vaginal biota in different regions of the world and how it impacts vaginal health. Because of the current study's limitations, metagenomic research to identify the whole vaginal microbiome may be performed in the future. Our results will need to be verified in a larger study utilizing molecular techniques and long-term research designs paired with data on participants' activities in order to further characterize this important defense and to help women suffering from vaginal ecosystem disruptions.

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Data availability statement

This published paper contains all of the data collected or evaluated during this investigation.

Contribution of the Author

BN conceived the presented idea, developed the theory, and wrote the manuscript with support from EY and DR. EM's encouragement to begin this work.

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Declarations

Conflict of interest: The authors state that no commercial or financial ties were present throughout the research. Therefore, a conflict of interests does not exist.

Ethical approval

The individuals who provided us with their samples for this study had given their consent. There were no animals involved in this study.

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Enhanced cell Viability with Induction of Pathogenesis Related Proteins Against *Aspergillus niger* in Maize by Endo-Rhizospheric Bacteria

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Abstract

Two endorhizospheric bacteria were isolated from the root of saline tolerant plant *Suaeda nudiflora* wild mosque by using nitrogen free selective medium and identified as *Pseudomonas aeruginosa* and *Bacillus megaterium* by the various biochemical and molecular analysis. The isolates were assayed for the production of the secondary metabolite/antibiotics and also analyzed for its infection antagonizing potential against the pathogen *Aspergillus niger.* The growth promotion potential of the isolates was analyzed on maize plant for enhanced plant growth/yield by induction of chitinase, catalase production and reduced programmed cell death (PCD) for acquiring resistance against pathogen *Aspergillus niger.* Comparative analysis of secondary metabolite production was studied in healthy and infected maize by GCMS analysis. The results showed that isolates were able to induce defense enzymes prior to infection and its activity further increased on pathogenic interaction. The isolates also induced PR proteins as well as expression of catalase like gene in the infected plant. The isolated endorhizospheric bacteria also modulate the types and level of secondary metabolites in maize plant for the better survival of maize under stress.

Keywords: Endorhizospheric, Chitinase, Catalase, Programmed cell death, Secondary metabolite, GCMS analysis, Gene expression**.**

1. Introduction

Ecological factors like biotic and abiotic ones continuously effect the growth of plant in agricultural farms. A co-evolution takes place between plants and microbes, which establish synergistic and antagonistic relations with microbes and the plant pathogen. A multilayered process is mediated by the pathogen for its establishment; at the same time, plant-derived metabolites as sugars, lipo-polysaccharides and proteins are produced by the plant to develop resistant against such stress (Boyd *et al.,* 2013). Numerous self-governing and well-elaborated mechanisms have been developed by the plant pathogens to penetrate the plant cell and retrieving the plant cell contents. Zandalinas *et al.,* (2017) reported, that during infection the penetration of pathogens usually depends on the precise time-course of the pathogen interaction with the host plant cells, which initiate various biochemical reactions, resulting in production of reactive oxygen species (ROS), secondary metabolites, and PR proteins. Sometime all these mechanism employed altogether in combination by the plant, to activate specific defense mechanism against the pathogen infection. A plant, when assessed by the pathogens, will stimulate production of several biochemical compounds like the oxidation of phenolic compounds, accumulation of secondary metabolites and many mechanisms related with defense in plants, upsurge specially during fungal infections (Zhou *et al.,* 2016). The oxidation of phenolic compounds develops a tendency for the formation of insoluble complexes with proteins, which act as enzyme inhibitors or are responsible for the oxidation of toxic elements to inhibit the pathogen infection. The most imperative protective response of plants towards the pathogenic factors is the production of pathogenesis-related proteins (PR proteins). The pathogenesis-related proteins are the group of plant encoded proteins responsible for specific forms of resistance to pathogens and related stress (Jha, 2019). The hydrolytic proteins like chitinase and catalase have some basic property and have three main functions in protecting plant from pathogens. Firstly, the hydrolysis of the particular substrates are catalyzed by such enzymes, thus releasing biologically active oligosaccharides (elicitors and suppressors) proficient of managing the immune status of plant tissues. Secondly, as the main constitution of fungal pathogen cell walls is chitin, such an enzyme has the ability to hydrolyze the cell walls of pathogenic fungi. Third, it also modulates the concentration of defense enzyme like peroxides to control the pathogen spread and initiate programmed cell death (PCD) (Park *et al.,* 2018). In the healthy plant, the PR proteins are present in insignificant amount and are induced at certain stages of growth only. Programmed cell death is accountable for the removal of undesirable damaged/dangerous cells and is an important genetically controlled process. PCD is critically for different aspects of plant life cycle, and also involved in hypersensitive response (HR) for the biotic defenses toward plant

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pathogens attack. Many bacteria, fungi, protozoa, algae like numerous microorganisms reside together in the plant rhizosphere, among them bacteria are the most abundant. Being the most abundant microorganism in the rhizosphere, bacteria directly influence the plants metabolism and physiology. There is a tough competition among the rhizospheric bacteria and pathogenic microorganism for root exudates/colonization (Barriuso *et al.,* 2008). The pathogen-induced production of PR proteins and systemic response in the plants and induction of systemic response resulted in accumulation of PR proteins not only at pathogen invasive site, but systemically induced in entire plant. Maize production in the field is constrained by both abiotic and biotic stress factors. In the present study, we investigate the efficiency of endorhizospheric bacteria to develop resistance against the fungal pathogen *Aspergillus niger* in maize through induction of PR proteins, secondary metabolites, phenolic content and gene expression. The effect of endorhizospheric bacteria has been analyzed on the degree of programmed cell death in infected/uninfected maize plant.

2. Materials and Methods

2.1. Isolation and identification of bacteria

Bacterial strains were isolated from root of plant *Suaeda nudiflora* wild mosque by serial dilution techniques as our published method (Jha and Subramanian, 2014). The selection of bacteria for further experiment was done on the basis of their ability to solubilize phosphate (Chen *et al.,* 2014), produce siderophore (Ferreira *et al.,* 2019), modulate indole-acetic acid (IAA) (Mohite, 2013), gibberellins, and utilizes 1-aminocyclopropane-1-carboxylic acid (ACC) as sole nitrogen source (Polko and Kieber, 2019). Morphology and Gram properties were determined using a light microscope (1,000X) (Zeiss, Argentina S.A). Pellicle-forming ability and aerobic N_2-dependent growth were assessed in semisolid nitrogen free semi-solid medium (NFb) (Himedia, Mumbai, India) with different carbon sources. For this purpose, malate was replaced by fructose, glucose, glycerol or sucrose. All the isolates were biochemically characterized for utilization of metabolites and enzymes such as Urease, Lysine Decarboxylase, Ornithine Decarboxylase, Esculin, Adonitol, Rhamnose, Mannitol, Sorbitol, Cellobiose, Melibiose, Glucuronate, Mannose, Maltose, Trehalose, Indole, Malonate, Phenylalanine desaminase, Sucrose, 5-Ketogluconate, Palatinose, Galacturonate, Colistin, Coumarate, Tetra thionatereductase, α-Galactosidase, Indoxylphosphate, Raffinose, o- NitroPhenyl N-Acetyl β – D Glucosaminide, P- Nitrophenylβ - D Galactopyranoside, Oxidase tests as per the standard methods (Patra *et al.,* 2020).

2.2. Molecular identification of bacterial isolates

The molecular identification of the isolates were done by 16s rDNA analysis with 16s rDNA primers 16S F: 5'AGAGTTTGATCCTGGCTCAG3' and 16S R: 5'AGGTTACCTTGTTACGACTT3' for PCR amplification of the DNA, with1kb ladder BLUE from GeneON, followed by sequencing (Bangalore, GeNei). The DNA sequences were compared with the sequence obtained from the nucleotide database. The sequences were aligned with the CLUSTAL-W program, and evolutionary distances were generated. Alignment gaps and ambiguous bases were not taken into consideration for comparison. Phylogenetic trees were constructed using the neighbor-joining method and the maximum likelihood method in PHYLIP package.

2.3. Compatibility between bacterial isolates and fungus

Bacterial isolates were tested for its compatibility with each other by streaking parallel on Yeast Extract Glucose Agar medium and incubated in incubator at 28 °C. Compatibility was tested by overgrowth or inhibition of growth after 72 hrs of incubation. Antagonism between bacteria and fungus was in-vitro determined by inoculating both organisms on the same Yeast Extract Glucose Agar plate at 28 °C in incubator for a week, and antagonistic effects were tested by inhibition of growth of fungus by bacteria.

2.4. Antibiotic production ability assay

The antibiotic production ability of the selected isolates was assessed by extracting and testing toxicity of metabolites produced by them. The strains were grown for 48 hrs in 5 ml of N broth with glucose (2% w/v). The culture was centrifuged at 10,000 rpm for 10 min and filtrate was collected. The metabolites from the filtrates were extracted with an equal volume of chloroform. The metabolites were also extracted from pellet and pooled. The upper aqueous layer was discarded and to remaining chloroform phase, a pinch of sodium sulphate was added to dry off water. It was again centrifuged at 8,000 rpm for 10 min to palettes out sodium sulphate. The clear layer was discarded, and chloroform was removed by flushing air. The residue was re-dissolved in 200 ml of acetone, and 70 µl was spotted on to TLC plate (Silica gel 60 F254, 20*20 cm, 0.2 mm thickness, Merck). The plate was chromatogrammed using chloroform: acetone (9:1) as solvent system. Later the plates were observed under UV light at 254 nm. The metabolites were eluted and re-dissolved in acetone: water (1:10) 100 µl of eluted portions were centrifuged to pellet. The silica gel clear suspension was further analyzed for toxicity against test pathogen. The percentage inhibition of individual antibiotic produced by each strain was calculated with known concentration of antibiotics Streptomycin.

2.5. Plant inoculation and treatment

Certified seeds of maize variety GM4 were obtained from the Gujarat state seed cooperation and were inoculated with endorhizospheric bacteria (Jha and Subramanian, 2015). After 4 weeks of inoculation, plants were infected with *Aspergillus niger* by spore suspension with a spore load of 10^4 conidia ml^{-1}, which caused more than 75% infection under greenhouse condition. Plants inoculated with bacteria and infected or non-infected with fungus were transplanted in pot, having five transplants per pot holding 10 kg soil and allowed to grow without additional nutrient for 5 weeks at 20 to 25 °C in the greenhouse.

2.6. Effect on total phenolic content after inoculation and infection

The total phenolic content of leaf extracts was measured using colorimetric Folin–Ciocalteu method

(Kaur and Kapoor, 2002). The leaf extract (1ml) was mixed with 5ml of distilled water and 250 µl of 1N folin-ciocalteau reagent. The mixture was covered and allowed to stand for 3 min at 25°C. In this mixture, 1ml of saturated Na_2CO_3 and 1ml of distilled water were added. The mixture was incubated for 1 hr at 25°C to develop color and measured at 725 nm using spectrophotometer. Standard graph was prepared by using different concentration of phenol crystals.

2.7. Assay of defense enzymes

Plant leaf tissues were collected after 2 weeks of inoculation and infection with pathogen. Leaf samples were homogenized with liquid nitrogen in a pre-chilled mortar and pestle. One gram of leaf sample was homogenized with 0.1 M sodium citrate buffer (pH 5.0) at 4ºC. The homogenate was centrifuged for 20 min at 10, 000 rpm. The supernatant was used as a crude enzyme extract for enzyme assay.

2.8. Estimation of chitinase

One gram of leaf tissue was homogenized in 5 ml of 0.1 $molL^{-1}$ sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 g at 4 °C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared by taking 2 g of crab-shell chitin (Sigma, USA). It was slowly added to 35 ml of cold concentrated hydrochloric acid with vigorous stirring and placed at 4°C for 24 h. The mixture was filtered through glass wool into ethanol (200 ml) with rapid stirring. The resultant chitin suspension was centrifuged at 10,000 g for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral. The commercial lyophilized snail gut enzyme chitinase was used as standard for colorimetric assay. The reaction mixture consist 10 ml of 1 $molL^{-1}$ sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) in an eppendorf tube. After 2 h at 37°C, the reaction was stopped by centrifugation at 1000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 ml of desalted snail gut enzyme for 1 h. After 1 h the reaction mixture was brought to pH 8.9 by adding 70 ml of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice bath. After the addition of 2 ml of 3-(dimethylamino) benzoid acid (DMAB), the mixture was incubated for 20 min at 37°C and immediately thereafter the absorbance was measured at 585 nm. N-Acetyl glucosamine (GlcNAc) was used as a standard. The enzyme activity was expressed as nmolGlcNAc equivalents per min per mg of protein (Kumar *et al.,* 2018).

2.9. Estimation of Catalase (CAT) activity

CAT activity was assayed by Chioti and Zervoudakis, (2017) method by measuring the initial rate of disappearance of H_2O_2. The reaction mixture contains of 0.1 ml enzyme from plant source, 0.1 mM EDTA and 3% (v/v) H_2O_2 in 0.05 M Na-phosphate buffer (pH 7). The activity was expressed as µmol H_2O_2 consumed per min by taking optical density at 240 nm.

2.10. Analysis ofprogrammed cell death

Caspase-like activity was assayed in the maize leaves after 2 weeks of infection treatment using the method of Sueldo and van der Hoorn, (2017). The leaf (200 mg) was ground in liquid nitrogen into a fine powder and homogenized in 2 ml of assay buffer containing 100 mM Tris–HCl (pH 7.2), 5 mM $MgCI_2$,2m MEDTA, 10%(v/v) glycerol, 10 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). This mixture was centrifuged at 13,000 g for 30 min at 4 °C to obtain tissue extract, and 25 µl of the tissue extract was incubated in70 µl of assay buffer at 37 °C for 5 min, followed by addition of 10 µl of 5 mM N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) as substrate (dissolved in dimethyl sulfoxide) for caspase-like activity to a final concentration of 0.5 mM. A blank reaction was set up in which Ac-DEVDpNA was substituted with 10 µl of DMSO. These reaction mixtures were incubated at 37 °C for 60 min, within which caspase-like activity was followed by measuring absorbance at 405 nm every 20 min during the 60-min incubation period. Caspase-like activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for the p-nitroaniline.

2.11. Agarose gel analysis of DNA for programmed cell death

Hundred milligram of leaf tissue from each treatment was frozen in liquid nitrogen immediately after sampling and ground with a mortar and pestle to a fine powder. Isolation of DNA was performed using a DNeasy plant mini kit (QIAGEN, Mississauga, Ontario, Canada) according to the manufacturer's instructions. To observe DNA fragmentation, samples (0.5 µg ml^{-1} $lane^{-1}$, final concentration) were run on 1%ethidium bromide agarose gel at a constant 50 V.

2.12. GCMS analysis for secondary metabolites production

Analysis by GC-MS was performed using a Thermo Gc–Trace Ultra Ver: 5.0, Pyrolysis auto sampler interfaced to a Perkin Elmer Turbomass Gold equipped with a fused silica capillary column (J & W; DBI; 30m length x 0.25 mm id. film thickness 0.25 µm). The fraction was pyrolysed at 610 °C and then introduced to the GC column. The transfer line was held at 280 °C and the source temperature was maintained at 180 °C and ionization energy was set at 70eV. Helium was employed as carrier gas (1 mL/m). The GC oven temperature was programmed: The column held initially at 70 °C/ m (isothermal) and then increased by at 8 °C/m to 260°C/ m min^{-1} (isothermal). Qualitative identification of the different constituents was performed by composition of the relative retention times and mass spectra with those of authentic reference compounds by retention indices (RI) and mass spectra. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns (Jha *et al.,* 2014).

2.13. RNA extraction and RT-PCR from isolated RNA

The bacterized plant material without infection was used for the isolation of total mRNA and the RT-PCR was done using a GeNei RT PCR Kit with certain modifications related with cyclic variation in time. 100 ng mRNA was incubated at 65°C for 10 min at room temperature, subsequently with oligo (T) primers, 0.25 mM deoxynucleotide triphosphates (dNTP's), 5 mM 1,4-dithiothreitol (DTT), 4 mM Tris-HCl pH 7.5, 20 mM KCl,

0.02 mM EDTA, 0.1% (v/v) Polyoxyethylene sorbitanmonolaurate (Tween 20), 0.5 µl M-MULV reverse transcriptase and 9µl nuclease free water for the synthesis of cDNA. The mixture was incubated at 37 °C for 1 h. Temperature was then raised to 95 °C for 2 min to denature RNA-DNA hybrid. The catalase gene was amplified by using forward primer 5'-TTAATCAGCCATGGATCCt-3' and reverse primer 5'-TTAATCAGCCATGGATCCT-3' and documentation was done by Gel Documentation System (Alpha Digi Doc RT, USA). All PCR with different primer combinations were performed in duplicate.

2.14. Statistical data analysis

All the data were analyzed for significance using analysis of variance ANOVA followed by Fisher's least significant difference test (P 0.05), using SAS software (SAS Institute, Cary, NC, USA).

3. Results

Thirty-five bacterial isolates were obtained from the root of *Suaeda nudiflora* plant, collected from coastal region of Gujarat using semi-solid nitrogen-free medium (Nfb). Out of 35, two isolates were selected on the basis of their growth promotion efficiency, by analyzing their ability to solubilize phosphate, produce siderophore, indole-acetic acid (IAA), gibberellins, and utilizes ACC as sole nitrogen source. It was also analyzed for their ability to overcome or suppress infection by producing β-1, 3-glucanases and chitinases and was identified on the basis of biochemical tests and sugar fermentation behavior as described in Bergey's Manual of Determinative Bacteriology (Table -1).

The total genomic DNA was isolated from both isolates for molecular identification and amplified by 16S rDNA specific primers. Nucleotides homology and phylogenetic analysis of the isolate was used for identification and isolate was identified as as *Pseudomonas aeruginosa* (Gene Bank Accession Number: JQ790515) and other as *Bacillus megaterium* (GeneBank Accession Number: JQ790514). The two bacterial isolates were compatible to each other as observed on nutrient plate. *B. megaterium* and *P. aeruginosa* were also tested for its compatibility with the phyto-pathogenic fungus *A. niger* , and results indicate that both isolates were remarkably suppressed the growth of fungus in its vicinity. A clear zone of inhibition was observed around the both *B. megaterium* and *P. aeruginosa* and it was larger in *P. aeruginosa* than the *B. megaterium* (Fig.1).

The ability of the isolates for the production of antibiotic showed that the *P.aeruginosa* (S1) had more variation in bands of antibiotic in compared to *B. megaterium* (R1-R4) on TLC plate. Antibiotic(s) produced by *P.aeruginosa* (S1) do not migrate on TLC plate and that fraction does not show any zone of inhibition, so it may be some other metabolite having no effect on pathogen, while antibiotic produced by *B. megaterium* (R1-R4) were different Rf value and showed significant variation in zone of inhibition. The isolates fraction of antibiotic on TLC were analyzed for their efficacy against *A. niger* and most of the antibiotics fraction showed the efficacy against *A.niger* (Table-2) by formation of zone of inhibition.

Table 1.Biochemical properties of the isolates to be used as bio-control agent.

S.No	Biochemical Tests	*B.megaterium*	*P.aeruginosa*
1	Urease	-ve	-ve
2	Lysine Decarboxylase	-ve	-ve
3	Ornithine Decarboxylase	-ve	-ve
4	Esculin	-ve	+ve
5	Arabinose	-ve	-ve
6	Adonitol	-ve	-ve
7	Rhamnose	-ve	-ve
8	Mannitol	-ve	-ve
9	Sorbitol	-ve	-ve
10	Cellobiose	-ve	-ve
11	Melibiose	+ve	-ve
12	Glucuronate	-ve	-ve
13	Mannose	-ve	+ve
14	Maltose	+ve	+ve
15	Trehalose	-ve	+ve
16	Indole	-ve	+ve
17	Malonate	-ve	+ve
18	Phenylalanine desaminase	-ve	-ve
19	Sucrose	+ve	+ve
20	5 Ketogluconate	-ve	-ve
21	Palatinose	-ve	-ve
22	Galacturonate	-ve	-ve
23	Colistin	-ve	+ve
24	Coumarate	-ve	+ve
25	Tetra thionate reductase	-ve	-ve
26	α- Galactosidase	-ve	-ve
27	Indoxylphosphate	-ve	-ve
28	Raffinose	-ve	-ve
29	O-Nitro phenyl N-Acetylβ-D Glucosaminide	-ve	-ve
30	p- Nitrophenyl β-D Galatopyranoside	-ve	+ve
31	Oxidase	-ve	-ve
32	Phosphate Solubilization	+ve	+ve
33	Siderophore production	+ve	+ve
34	Indoleacetic acid (IAA)	+ve	+ve
35	ACC Utilization	+ve	+ve
36	β-1, 3-glucanases	+ve	+ve
37	Chitinases	+ve	+ve

Note- The +ve sign indicate presence and –ve sign indicate absence of activity.

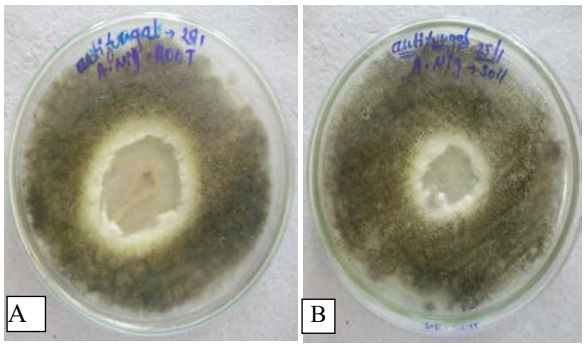

Figure 1. Endophytic bacteria showing antifungal activity against *A.niger* as the formation of Zone of clearance around the culture A= *P. aeruginosa* and B= *B. megaterium*.

The efficacy of the isolated antibiotics (from TLC) had been determined by comparing the zone of inhibition with known standard antibiotics (streptomycin). The zone of inhibition the isolated fractions of antibiotics from the TLC plate showed remarkable variation and the highest efficacy (73%) was shown by the fourth fraction of antibiotics of *P. aeruginosa*.

Plant phenols are secondary metabolites that encompass several classes of structurally diverse products arising from the shikimate–phenylpropanoid pathways. Plants use phenolic compounds for pigmentation, growth, reproduction, and resistance to pathogens. In the present study, the endorhizospheric bacteria considerably enhanced the phenolic compounds in plant under both conditions, i.e. inoculated with isolates alone and infected with the *Aspergillus niger*. The endorhizospheric bacteria alone able to induced production of sufficient amount the phenolic in inoculated plant, while when such inoculated plant get infected with fungal pathogen *Aspergillus niger,* enhanced production of the phenolic was recorded as shown in Table 2.

Table 2. Analysis of antibiotic(s) production by endophytic bacteria on TLC and their efficacy against *A. niger.*

Endophytic Strain	Antibiotic	Rf values	Zone Of Inhibition, ZOI (mm)	Antibiotic efficacy (%)
B. megaterium	S1	Absent	Absent	00
P.aeruginosa	R1	0.20	12	67%
P.aeruginosa	R2	0.515	8	54%
P.aeruginosa	R3	0.656	Absent	00
P.aeruginosa	R4	0.781	15	73%

In the present study, the protective enzymes considerably enhanced after inoculation with endorhizospheric bacteria in the plant and infection by *Aspergillus niger,* further changed in its activity. A simultaneous increase was recorded in chintase and catalase activity in the plant inoculate with endorhizospheric bacteria alone or in mixture and infected with fungal pathogen *Aspergillus niger.* Chitinase belong to the glycosyl hydrolase family, which catalyzes the hydrolysis of glycosidic bonds of chitin.

However, the CAT activity was significantly decreased in bacterized plant up to (22.2 nmol/min/g) at normal condition, but after infection with pathogen its concentration further increased upto (38.2nmol/min/g) (Table 3). So, the CAT activity remarkably increased after fungal infection in non-inoculated plants compared to plants inoculated with endophytic bacteria.

Table 3: Effect of endophytic bacteria on the phenolic, catalase and chitinase activity in maize after infection (n=5).

Treatment	Phenolic (mgg^{-1} of the gallic acid equivalent)	CAT (nmol $min^{-1}g^{-1}$)	Chitinase (nmol of NAG $min^{-1}g^{-1}$)
	Normal		
	m mm		
Control	0.93^{cd}	0.34^{d}	0.21^{d}
Control+*B. megaterium*	1.61^{bc}	0.47^{bc}	0.28^{c}
Control + *P.aeruginosa*	1.88^{ab}	052^{b}	0.27^{b}
Control+ *B.megaterium*+ *P.aeruginosa*	2.11^{a}	0.61^{a}	0.34^{a}
Infected			
Control	1.21^{cd}	0.83^{d}	0.29^{d}
Control+*B. megaterium*	1.87^{c}	0.53^{ab}	0.37^{b}
Control + *P.aeruginosa*	2.14^{b}	0.51^{a}	0.41
Control +*B.megaterium*+ *P. aeruginosa*	2.45^{a}	0.41^{c}	0.51^{bc}
Signification	**	*	ns

$^{ns}P > 0.05$ (not significant), $*P < 0.05$ (significant) and $**P < 0.05$ (highly significant), m- mean. For each Parameter, values in columns followed by the same letter are not significantly different at ($P \leq 0.05$).

GCMS analysis of leaf extract of inoculated and non-inoculated plants after infection showed significant difference in the types and number of secondary metabolites and the results revealed the presence of different phytocompounds in the plant inoculated with selected bacteria. The healthy-inoculated plant showed 8 secondary metabolites (Table 4), while plant inoculated and infected showed 12 different secondary metabolites (Table 5) belong to phenols and terpenoids group. The compounds like androstane-3,17-dione, methyl dihydroisosteviol, isosteviol methyl ester in healthy and androstan-17-one, 3-ethyl-3-hydroxy-, (5.alpha.), 1h-3a,7-methanoazulene, octahydro-1,4,9,9-tetramethyl, aromadendrene oxide-(2) belong to phenols and terpenoids group in infected plant.

Fungal infection can trigger activation of caspase-like activity in the infected leaves. On this basis, we investigated the level of caspase-like enzymatic activity differed in maize plant inoculated with endorhizospheric bacteria and infected with fungal pathogen *Aspergillusniger.* Cell caspase-like enzymatic activity increased for both the inoculated plant with endophytes and infected with pathogen and more in non-inoculated infected plants (Fig. 2).

Table 4. Different secondary metabolites in endophytes inoculated maize leaves extract identified in GC–MS analysis.

Hit	Rev	For	Compound Name	MW	Formula	CAS
1	812	565	2,4-DIBROMOETIOCHOLA-3,17-DIONE	444	C19H26O2Br2	900251-89-1
2	787	576	ANDROSTANE-3,17-DIONE	288	C19H28O2	5982-99-0
3	750	512	ISOSTEVIOL METHYL ESTER	332	C21H32O3	900256-08-8
4	741	521	1,3,6-HEPTATRIENE, 2,5,5-TRIMETHYL	136	C10H16	29548-02-5
5	724	489	METHYL ISOLITHOCHOLATE	390	C25H42O3	5405-42-5
6	720	509	METHYL DIHYDROISOSTEVIOL	334	C21H34O3	202577-02-4
7	715	538	ALPHA.-FARNESENE	204	C15H24	502-61-4
8	714	478	TRILOSTANE	329	C20H27O3N	13647-35-3

Where hit means attempt Number, Rev Reverse match of peak, For Forward match of Peak, M.W. Molecular weight of compound, CAS Chemical Abstract service.

Table 5. Different secondary metabolites in endophytes inoculated maize leaves extract identified in GCMS analysis after fungal infection.

Hit	Rev	For	Compound Name	M.W	Formula	CAS
1	798	601	3-KETO-ISOSTEVIOL	332	C20H28O4	900255-38-4
2	779	561	3-HYDROXY-12-KETOBISNORCHOLANIC ACID	362	C22H34O4	900252-01-9
3	749	553	3-HYDROXY-11-KETOCHOLANIC ACID	390	C24H38O4	910-28-1
4	738	543	1-PROPENE, 2-NITRO-3-(1-CYCLOOCTENYL)	195	C11H17O2N	80255-21-6
5	722	531	PSEDUOSARSASAPOGENIN-5,20-DIEN	414	C27H42O3	900214-84-5
6	715	538	ALPHA.-FARNESENE	204	C15H24	502-61-4
7	714	478	TRILOSTANE	329	C20H27O3N	13647-35-3
8	713	425	ANDROSTAN-17-ONE, 3-ETHYL-3-HYDROXY-, (5.ALPHA.)-	318	C21H34O2	57344-99-7
9	703	506	1-NAPHTHALENECARBOXYLIC ACID, DECAHYDRO-1,4A-DIMETHYL-6-METHYLENE-	316	C21H32O2	10178-35-5
10	703	522	1H-3A,7-METHANOAZULENE, OCTAHYDRO-1,4,9,9-TETRAMETHYL	206	C15H26	25491-20-7
11	702	501	AROMADENDRENE OXIDE-(2)	220	C15H24O	900151-98-6
12	699	537	GAMMA.-ELEMENE	. 204	C15H24	339154-91-5

Where hit means attempt Number, Rev Reverse match of peak, For Forward match of Peak, M.W. Molecular weight of compound, CAS Chemical Abstract service.

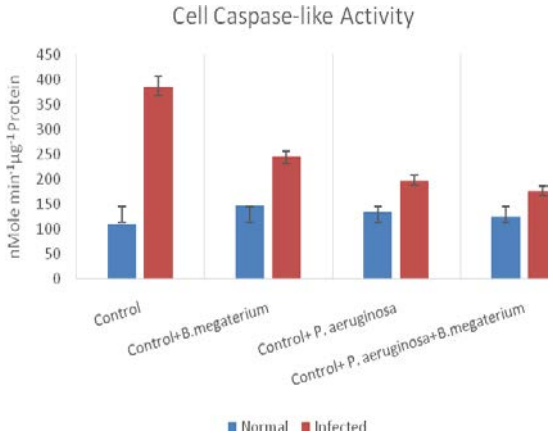

Figure 2. Effect of inoculation with *B. megaterium*, *P. aeruginosa* alone and in combination on cell caspase-like activity in maize in normal and infected condition (n=5).

Genomic DNA was isolated from leaves of all different treatment and separated in agarose gel electrophoresis to analyze programmed cell death (Fig. 3). Fungal infection showed extensive DNA fragmentation as detected in both the inoculated or non-inoculated maize. Less fragmentation of DNA was observed in the plants inoculated with endophytes only. To characterize the induction of catalase gene in maize inoculated with endorhizospheric bacteria, the RT-PCR-amplified product was observed as1.8 kb bands on agarose gel electrophoresis (Fig. 4) uniformly by inoculated with either single isolate or in combination.

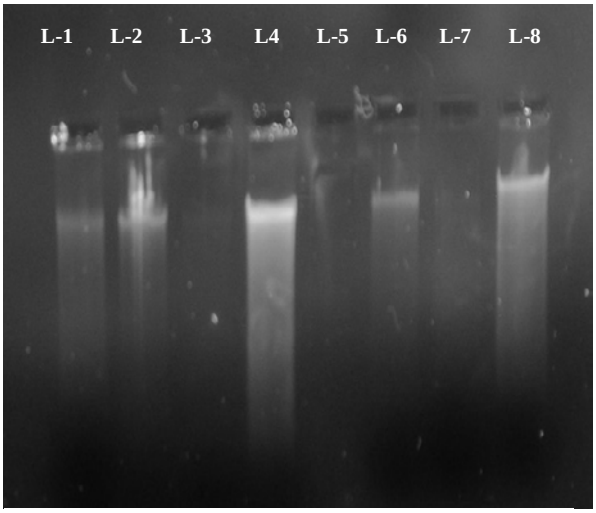

Figure 3: Effect of *B. megaterium*, *P. aeruginosa* alone and in combination on programmed cell death in in maize in healthy and infected condition (n=5). Where L1-infected inoculated with both the isolates, L2- infected inoculated with *B. megaterium,* L3-infected inoculated with *P. aeruginosa,* L4- Infected Control, L5-Pure control, L6- inoculated with both the isolates, L7-inoculated with *B. megaterium*, L8- inoculated with *P. aeruginosa*.

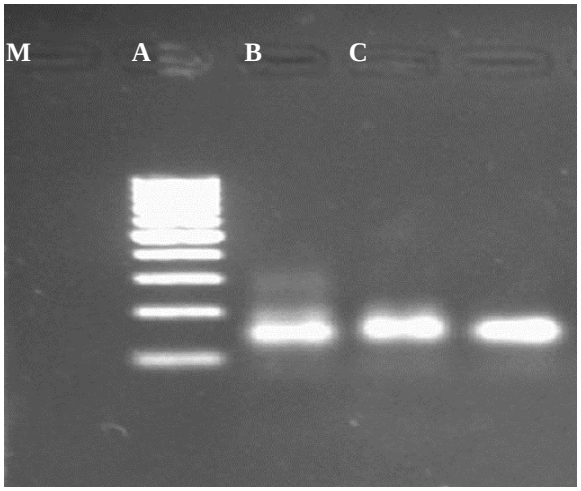

Figure 4. Agarose gel showing induction of gene catalase. Lane M=1kb *ladder* BLUE from GeneON Bioscience is composed of 13 individual *DNA* fragments , Lane A= plant inoculated with *B. megaterium,* and Lane B= plant inoculated with *P. aeruginosa,* Lane C= plant inoculated with *B. megaterium* and *P. aeruginosa.*

4. Discussion

Many interactions of microbes with plants takes place in the endosphere, due to the occurrence of rich and diverse microbial food source, so it is the most active ecological niches, (Jha and Subramanian, 2016). Such interaction of plant with endorhizospheric bacteria helps in maintaining the plant health by improving nutrient status, developing resistant against phyto-pathogen and tolerance towards the environmental stress. The plant root colonized by endorhizospheric bacteria has numerous mechanisms to employ favorable effect on plant growth and development, i.e. by associative nitrogen fixation, solubilization of phosphorus and potassium, production of siderophores, and by modifying the cell permeability (Qessaoui *et al.,* 2019). Such isolates also enhance bioavailability of nutrients, modify root proton efflux, directly motivate plant ion uptake and/or plant transport system and enhance the overall biomas production. In this study, *Pseudomonas aeruginosa* and *Bacillus megaterium*, were two bacterial isolates obtained from root of *Suaeda nudiflora wild mosque* plant, and it is well reported that bacterial genera such as *Pseudomonas, Bacillus* and *Brevibacillus* are well known to endorse plant growth and yield in diverse groups of non-leguminous plant under normal as well as stress conditions (Karlidag *et al.,* 2007).

Antibiotics are generally low molecular weight organic compounds produced by microbes, to inhibit the growth of other microbes. Antibiosis plays an active role in the bio-control of plant disease and often acts in concert with competition and parasitism. The result of present study showed that the antibiotic produced by the isolates has the potential to suppress the growth of fungal pathogen and ultimately help in plant growth promotion. Olanrewaju *et al.,* (2017) has also been reported that *Bacillus* and *Pseudomonas* produce a variety of antibiotic metabolites which serve as antifungal, antibacterial, antihelminthic, antiviral, antimicrobial, phytotoxic, antioxidant and cytotoxic agents. The bio-control potential and induced response of these isolates is an effective tool for yield management in economically important crops.

The phenolics normally produced by the plant to strengthen the plant cell wall also have fungicidal activity. In the present study, healthy as well as maize plants infected with fungal pathogen showed better induction of phenolic compounds after being inoculated with endorhizospheric bacteria. Induction/accumulation of phenolics takes place prior to pathogens attack or pathogen infection to protect the plant from pathogens as well as to check the complete hold pathogen on the host plant (Nicaise *et al.,* 2009). In this study, production of phenolics induced in the endophytes inoculated maize plant helps the plant to overcome phytopathogenic infection. The results of the present study showed higher induction of phenolic compounds in the plants colonized with beneficial microorganisms after pathogen infection, compared to healthy controls and are responsible for inducible protection in plant, to maintain plant health for much longer time as also reported by Wallis and Galarneau, (2020).

In response to biotic stress, plants implement a vast range of mechanisms like modification of specific metabolites/up regulation of defense-related proteins to develop resistant over pathogen. Pathogenesis-related proteins include a huge family of plant proteins, which induced in response to pathogen invasion and are developmentally controlled. Pathogen induced SAR is similar to endorhizospheric bacteria mediated ISR to develop resistant towards a wide range of plant pathogens and enhance resistant to uninfected plant parts also (Choudhary *et al.,* 2008). Induction of defence enzymes chitinase and catalase take place more efficiently in maize plant inoculated with endorhizospheric bacteria, to develop resistant against the phytopathogens. Chitinase is an important hydrolytic enzyme, which has been induced frequently in plants upon infection by diverse group of pathogens. The amount of chitinase considerably increases in the maize plant inoculated with endophytes in this study and has an effective role in plant defense against fungal pathogen, as it is responsible for degradation of fungal cell wall. Daulagala and Allan- Atkins, (2015) reported, about the chitinolytic activities of endophytic bacteria isolated from symptom-free chinese cabbage leaves, which clearly indicated that the induction of defence enzyme by such bacteria alone is sufficient for the host plant protection. The main structural component of fungal cell wall is chitin, substrate for chitinase enzyme. Pathogen infection in plant can result in generation of reactive oxygen, which has direct impact as antimicrobial action and plays critical role in inducing various other defense mechanisms, like production of phyto-alexin, lignin deposition, peroxidation of lipid and hypersensitive cell death or PCD.

Catalase is an essential enzyme have major role in plant defense against pathogens. In this study, plant inoculated with endorhizospheric bacteria showed significantly enhanced catalase activity, but it reduced after infection by fungal pathogen. The reduced catalase activity may cause the suppression of H_2O_2 scavenging activity, perhaps has a major role in modulating H_2O_2 homeostasis, as accumulation of threshold levels of H_2O_2 is necessary for the activation of vigorous programmed cell death for pathogen clearance (Lapshina *et al.,* 2016). In the plant's life cycle, cell death is a crucial process, and two main

approaches for action in plants are programmed cell death (PCD) and necrosis. PCD is genetically controlled and has some common characteristic features with apoptotic cell death of the cell like cytoplasmic condensation, cell shrinkage and DNA fragmentation (Greenberg and Yao, 2004).

Pathogen induced host cell destruction observed as enhanced DNA fragmentation in infected plant and plant inoculated with endophytes showed reduced DNA fragmentation, which indicates the role of endophytic bacteria in managing cell activity. During the plant pathogen interaction, at the site of pathogen attack an oxidative burst occur with initiation of cell death at the site of pathogen attack and such localized cell death restrict the spread of the pathogen infection to the healthy plant cell/part.

In the GC-MS analysis there are eight different metabolites/compounds obtained in the leaf extracts of plant inoculated with endophytes alone and twelve different metabolites in infected plant inoculated with endophytes. Among these twenty different metabolites ten metabolites exhibit antioxidant, antibacterial and antimicrobial activity as also reported by Nivas and Boominathan, (2015). Several bioactive natural compounds having antimicrobial activity are induced by the endophytes, which help host plant to acquire resistant against pathogen. The pathogenic intrusion regulated by modulation of secondary metabolites by the endophytes, to develop resistance against pathogen in the host plants. Many such secondary metabolites having antimicrobial activity belong to diverse structural classes such as alkaloids, peptides, steroids, terpenoids, quinines, flavonoids and phenols are reported has been induced by endophytes (Yu *et al.,* 2010). In this present study, the production of diverse types and number of secondary metabolites in the endophytes inoculated plants in presence and absence of pathogen was observed during GCMS analysis.

Suppressing the fungal pathogen by initiating the plant defense mechanism by endorhizospheric bacteria is known as “Induced Systemic Resistance”. Plants immune system activates upon interaction of plant with endorhizospheric bacteria remain active throughout the life by inducing the defense gene. The endophytes alone are able to induce the defense gene like catalase in the plant as observed in the present study. Ramos Solano *et al.,* (2008) also reported about the induction of defense-related marker genes by some strains of *Chryseobacterium* and *Bacillus* in host plant. The plant growth and yield in the radish, potato, sugar beet, ground nut and mango increased by the *Pseudomonas,* in addition to disease management has been reported by Liu *et al.,* (2017). The major effects of inoculation of plant with such bacteria may be similar to treatment with pathogenic microorganisms or abiotic agents, ensued in the induction of systemic resistance for bacterially induced resistance and systemic acquired resistance for the other forms. The development of bio-agents using such bacterial strains is an emerging trend in crop protection to reduce the economic loss caused by pathogen.

5. Conclusion

The endorhizospheric bacteria having ability for the induction of phenolic production, PR protein and secondary metabolites with antimicrobial activity in the host plant, can serve as a potential biocontrol agent against wide range of phytopathogen. Such secondary metabolites belong to diverse structural classes such as alkaloids, peptides, steroids, terpenoids, quinines, flavonoids and phenols. The present study strongly supports the development of bio-control strategies on the basis of *invitro* effect of the isolates against the fungal pathogen and production of other biologically active metabolites using such endorhizospheric strain, to reduce the damage caused by plant pathogens in economically important crops.

Conflict of Interest

There is no conflict of interest to declare.

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Characterization, Biofilm and Plasmid Curing Effect of Silver Nanoparticles Synthesis by Aqueous Extract of *Myristica fragrans* Seeds

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Abstract

Nowadays nanoparticles are used in many fields of life all over the world, and there are numerous ways to obtain them: chemical, physical and biological processes. In recent times, the biological method for the synthesis of nanoparticles associated with using plant extract is widely spread.

Optimal conditions for synthesis of silver nanoparticles using aqueous seeds extract of *Myristica fragrance* were highlighted in this research, such as type of plant extract, weight of extracted plant material, volume ratio of plant extract to $AgNO_3$ and temperature of reaction.

The study proved that the optimal status for AgNPs synthesis by using 10 g of *M. fragrance* seeds powder were added to 100 mL boiled distilled water, then homogenized and filtered after 24 hours. Aliquot of 5 mL of hot aqueous extract were added to 45 mL of $1*10^{-3}$ M $AgNO_3$ solution in the water bath with a magnetic stirrer for the bio-reduction process at 60 °C. The biological activity of AgNPs nanoparticles was evaluated by using well diffusion method and biofilm formation for G+ and G- bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Klebsiella pneumonia*, while the effect of AgNPs nanoparticles on plasmid curing was investigated for *Escherichia coli* and *Staphylococcus aureus* only.

Atomic Force Microscopy (AFM) images proved that Ag particles are in nanometer- size and have granular shape, the size of silver nanoparticle is (74.55 nm) for the sample taken after 16 min of the reaction.

Nanoparticles of various concentrations have proven effective in inhibiting bacterial growth after antimicrobial activity test, biofilm formation and plasmid curing as they exhibited a remarkable effect in inhibiting the growth of both Gram-positive and negative bacteria.

Keywords: silver nanoparticles, green method, biofilm, plasmid curing, *Myristica fragrans*,

1. Introduction

Nanotechnology today is one of the most interesting fields of sciences because of its multifunctional properties, specifically the size range from (1-100) nm (Zia *et al.,* 2016 and Sithara *et al.,* 2017).

The unique size of nanoparticles allowed using them in various fields of applications such as medicine, food, biomedical, electronics, mechanical industries, biotechnology and environmental because of their many properties (Ahmed *et al*., 2016; Khatoon *et al*., 2017 and Henríquez *et al*., 2020). The properties of NPs relay on the specific parameters like shape, size and surface of molecules; while achieved nanoparticles quality is based on their synthesis methods (Kuppusamy *et al.,* 2016 and Sithara *et al.,* 2017).

Song and Kim (2009) reported on the synthesis of Ag nanoparticles by the reduction of Ag+ using Pine, Persimmon, Ginkgo, Magnolia and Platanus leaves broth. The time courses of AgNPs synthesis with different reaction temperatures were studied.

The best reducing agent in the duration of synthesis rate and diversion to silver nanoparticles was Magnolia leaf extract, where the AgNPs was synthesized during 11 min of reaction time and a reaction temperature at 95 °C.

Heydari and Rashidipour (2015) studied the effect of different concentrations of Jaft aqueous extracts (*Quercus infectoria*) on the synthesis of silver nanoparticles; the results showed that increasing the concentration of Jaft aqueous extract led to synthesize more silver nanoparticles.

The classical methods for nanoparticles formation are chemical and physical methods, but they are not eco-friendly because of using many toxic chemical compounds (Carrillo-Lopez *et al*., 2016 and Kambale *et al*., 2020). Today, the green method includes the use of plant extracts which is preferable over other methods as they provide reducing agents of secondary metabolites such as flavonoids, alkaloids, phenolics, tannins and terpenoids. Availability of plant sources makes this method easy and

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cost effective (Carrillo-Lopez *et al.,* 2016; Sithara *et al.,* 2017 and Khan *et al*., 2018).

The Ag nanoparticles are considered as one of the important metallic nanoparticles because of their unique characteristics like catalytic activity, chemical stability and good conductivity, so it is commonly used as anti-bacterial, antifungal, anti-viral and anti-inflammatory (Zhang *et al*., 2014; Ibrahim, 2015 and Liao *et al*., 2019). In this study, *Myristica fragrance* seeds called nutmeg belonging to the Myristicaceae family were used for Ag nanoparticles formation.

This aromatic plant has a wide range of active phytochemicals such as alkaloids, terpenoids, phenolic, lignins, tannins, steroids, flavonoids, etc. (Saxena and Patil, 2012; Bindu and Kumar, 2013 and Gupta *et al.,* 2013). The aim of this study is to identify the optimal conditions of AgNPs synthesis such as type of plant extract, temperature, time and the concentration of Ag NO_3 to achieve the best concentration of nanoparticles.

2. Materials and Methods

2.1. Preparation of cold, boiling and hot M. fragrance aqueous seed extracts

M. fragrans seeds were bought from local market in Baghdad, grinded in electrical grinder for 5-7 min, then 10 g of seeds powder was added to 100 mL of boiling distilled water to prepare hot aqueous extract, while to prepare cold aqueous extract, 10 g of seed powder was soaked in 100 mL distilled water.

A quantity of 10 g of *M. fragrans* seeds powder was added to 100 mL distilled water then heated for 1 hour at 95 °C to prepare the boiling aqueous extract. The three extracts were homogenized by using magnetic stirrer for 3 hours then left overnight at 4 °C. The extracts were filtered using Whitman paper No.1 then centrifuged at 8000 rpm for 10 min and stored at 4 °C until use (Banerjee *et al*., 2014 and Owaid *et al*., 2018).

2.2. Preparation of silver nanoparticles using plant extract

AgNPs was synthesized by adding 45 mL of $1*10^{-3}$ M of $AgNO_3$ (Sigma-Aldrich) solution in a dark flask placed in a water bath with stirring continuously till the temperature stabilized at 60 °C then 5 mL of seed extract was added (AL-Azawi *et al.,* 2018), 2 mL was taken from the reaction mixture every 2 and 5 min for 20 and 30 min respectively. All samples were measured using UV spectrophotometer; gradually, the change of color to dark brown from light yellow was observed after 2-3 days.

2.3. Characterization of AgNPs

2.3.1. UV spectrophotometer measurement

Preliminary characterization of the AgNPs was carried out using UV-vis spectroscopy. The UV-vis absorption spectra of the AgNPs were measured at room temperature on a spectrophotometer (UV-Spectrophotometer-Shimadzu UV-1800) in 1 cm optical path quartz cuvette. The optical behavior of the biosynthesized AgNPs aliquots of samples were analyzed every 2 min in the range of a wavelength from 190 - 1100 nm (Jain and Mehata 2017).

2.3.2. Fourier Transforms Infrared Spectrophotometer (FTIR) measurement

FTIR measurements were carried out to identify various functional groups in *M. fragrans* extract which are responsible for reducing and stabilizing the synthesized AgNps. The samples of AgNPs and *M. fragrans* extract were prepared by dropping 3 mL from each sample directly on a glass slide and left to dry at room temperature. The dried samples were grinded with KBr and analyzed on using Shimadzu IR-Affinity^{-1}. (Shanan *et al.,* 2018 and Fatema *et al.,* 2019).

2.3.3. Atomic Force Microscopy (AFM) measurement

The surface morphology of AgNPs was studied by using AFM (Angstrom AA2000, contact mode, atmospheric conditions, USA) images, which clarifying topological images at high magnification of surface morphology. Aliquot of 0.5 mL of AgNPs sample was centrifuged using eppendrof tube for 5 min at 10000 rpm. A few drops from the sample were placed on the slide, air dried and characterized by using atomic force microscopy (Karoutsos, 2009 and Carapeto *et al*., 2019).

2.4. Identification the optimal conditions for synthesis silver nanoparticles

2.4.1. Evaluation of the optimal temperature in AgNPs synthesis

The optimum temperature for synthesis of AgNPs was determined by added 45 mL of $1*10^{-3}$ M $AgNO_3$ solution to 5 mL of hot aqueous extract of *M. fragrans* in a flask placed in a water bath with continuous stirring at different temperatures (40, 50, 60 and 70) °C every time, 2 mL was taken from the reaction mixture every 2 min for 20 min; all samples were measured using UV spectrophotometer immediately and after 15 days (Verma and Mehata, 2016).

2.4.2. Evaluation of the ratio of hot aqueous plant extract to $AgNO_3$ solution in AgNPs synthesis

Different volumes of hot aqueous plant extracts and $AgNO_3$ solution were used in synthesis of AgNPs by adding 45, 25 and 5 mL of $1*10^{-3}$ M $AgNO_3$ to (5, 25 and 45) mL of hot aqueous plant extract respectively. All the samples were placed in a water bath at 60 °C with continuous stirring then measured using UV spectrophotometer (Ibrahim, 2015 and Ahmed *et al.,* 2016).

2.4.3. The effect of difference in plant material weight on the preparation of M. fragrans hot aqueous extract in AgNPs synthesis

The hot aqueous extracts of *M. fragrans* were prepared using different weights of seeds powder (6, 8 and 10) g then extracted in 100 mL of boiling distilled water. AgNPs was synthesized by adding 45 mL of $AgNO_3$ solution to 5 mL of plant extract every time of reaction as mentioned above; 2 mL was taken from the reaction mixture every 5 min for 20 min then all samples were measured using UV spectrophotometer (Sithara *et al*., 2017).

2.5. Biological activity

2.5.1. Antimicrobial activity

The antimicrobial activity of silver nanoparticles was investigated using the well diffusion method on Mueller-Hinton agar (MHA) (Merck). The inhibition zones were

reported in millimeter (well size was 6 mm) against various sorts of bacteria like gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*) and gram positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*).

MHA agar plates were inoculated with bacterial strain under aseptic conditions and wells were filled with 50 µL at various concentrations of AgNPs (100, 75, 50 and 25) % and incubated at 37 °C for 24 hours. After the incubation period, the inhibition zones were measured (Ibrahim, 2015).

2.5.2. Determination of minimum inhibitory concentration (MIC)

AgNPs was used to determine MIC for planktonic cells of *E. coli, K. pneumonia, S. aureus* and *S. epidermidis* bacteria. MIC test was conducted in 96- well microtiter plate; each test well was filled with 100 µL Trypton soya broth (TSB) (Merck) + 1% w/v glucose (BHD). Aliquot of 100 µL of AgNPs was added to the first test well then mixed well to prepare a double series of dilutions across the plate.

Aliquot of 10 µL of bacterial suspension was placed in each well plate to achieve a final inoculum size of $4x10^5$ CFU / mL. The positive control was achieved by adding TSB and bacterial inoculum without adding AgNPs to the well. Negative control was achieved by adding AgNPs without inoculum. After incubation for 18- 24 h at 37 °C, the microbial growth in the well was examined by turbidity. The MIC value was described as the lowest AgNPs concentration inhibits 80% of microbial growth, relative to the negative and positive controls (Christensen *et al*., 1995).

2.5.3. Biofilm activity

Biofilm formation test was achieved by using 96- well microtiter plate (Sánchez *et al.,* 2016). *E. coli, K. pneumonia, S. aureus and S. epidermidis* bacteria were cultured in Trypton soya (TS) broth for 24 h then the resulting culture was diluted to 1:100 TSB (Merck) + 1% w/v glucose (BHD). Each well of microplate was loaded with 100 µL of TSB and 100 µL of AgNPs produced from 12 and 16 min except the control well without AgNPs, then microplate, was incubated at 37 °C for 24 h, and each concentration was carried out in triplicate.

The planktonic bacteria were removed by washing the microtiter plate with sterile distilled water for 3 times; subsequently, 0.1% w/v crystal violet solution was added to each well then left to stain for 10 min at 25 °C. The crystal violet dye was removed by immersing the plate in a water tray, then inverting on paper towels to remove excess liquid and left to air dry. The plate was treated with ethanol 96% for 10-15 min at 25 °C to solubilize the dye bound, and then absorbance was measured at 630 nm (AL-Azawi *et al.,* 2019).

2.5.4. Plasmid curing

The bacterial DNA plasmid was extracted by using alkaline lysis method. The pathogenic bacteria *E. coli* and *S. aureus* were inoculated into 5 mL Nutrient broth (Merck) tubes after being treated with different concentrations (0.5, 1, 3 and 6) % of silver nanoparticles of the samples 12 and 16. All the tubes were incubated for 24 hours at 37 °C with shaking except the control tubes (without AgNPs). The extraction of plasmid DNA was carried out according to the Green and Sambrook, (2012) method.

3. Results and Discussion

Differences in physical and chemical conditions such as type of extraction, temperature, reaction period, weight of plant material and reactants concentration significantly affected the form, size and the morphology of Ag nanoparticles.

2.6. Visual observation of AgNPs

At the beginning of the reaction between *M. fragrance* extract and $AgNO_3$ solution, the reaction mixture was transparent, but over time the solution changed to light yellow and then to light brown. The chromatic change in the solution indicates the formation of AgNPs (Pirtarighat *et al.,* 2019). After about two weeks, the color of the solution turns dark brown with increasing color intensity as shown in Figure 1.

Increasing incubation time of the solution enhanced the growth of silver nanoparticle, and this result corresponds with the result of Ibrahim, 2015. Also, the presence of secondary metabolites in the plant extracts such as alkaloids and flavonoids that provide the electronic capacity of reduction Ag^+ to Ag^o as mentioned by Sharma *et al.,* 2014; Verma and Mehata, 2016; Izah *et al.,* 2018 and Khan *et al.,* 2018.

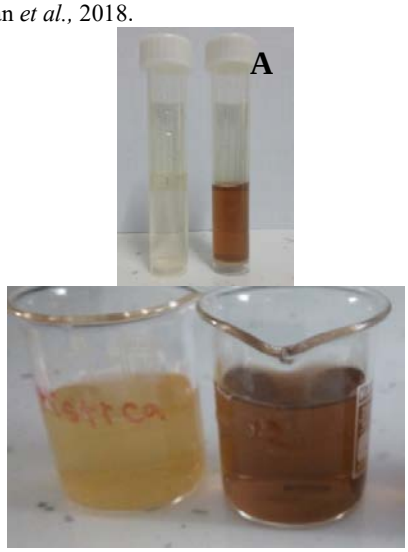

Figure1. The change in color of AgNPs solution over time after adding *M. fragrance* seeds extract to 10^{-3}M $AgNO_3$ (A) Left: 10^{-3}M aqueous silver nitrate. Right: $AgNO_3$ with *M. fragrance* seed extract forming AgNPs after two weeks. (B) Left: *M. fragrance* seeds aqueous extract 100 g/L. Right: 10^{-3}M $AgNO_3$ with the seed extract forming AgNPs after two months.

2.7. Characterization of AgNPs biosynthesis and the effect of reaction time on the formation of silver nanoparticles

2.7.1. UV–vis spectrophotometric analysis was carried out as first identify for AgNP formation.

Different components may contribute to the reduction of Ag ions during AgNPs biosynthesis by using plant extract. Figure 2 shows the visible spectrum of ultraviolet radiation for AgNPs obtained from the reaction of *M. fragrance* hot extract and 10^{-3}M $AgNO_3$ recorded 200 to 800 nm at regular intervals every two minutes. The maximum absorption observed and represented the extreme energy is 416 nm as obtained by Sharma *et al.,* 2014 and Ibrahim, 2015 showing that the synthesized of AgNPs are smaller with steady size distribution. It is noted from Figure 2 the absorption spectra due to the rapprochement of reaction time.

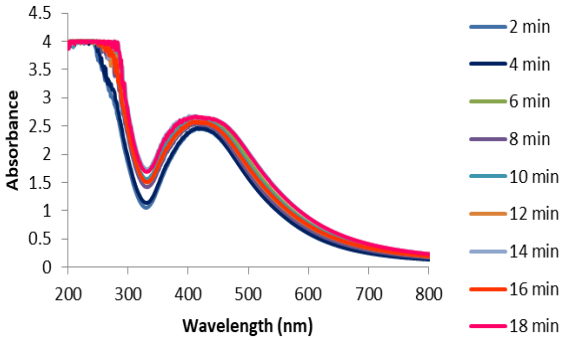

Figure 2. Absorption spectra of silver nanoparticales at various reaction time intervals of 10^{-3}M $AgNO_3$ with *M. fragrance* seed extract.

Time represents an important factor in the formation of silver nanoparticles. Figure 3 represents the absorption spectra of silver nanoparticles at 60 °C and at various reaction time intervals, every 5 min which was measured after 7 days of reaction. No visible peaks were obsereved.

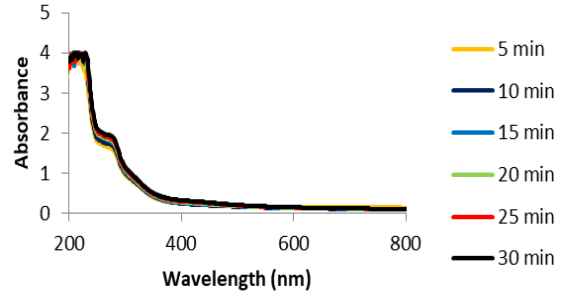

Figure 3. Absorption spectra of AgNPs at various reaction time intervals of *M. fragrance* hot aqueous extract and 10^{-3}M $AgNO_3$ at 60 °C after 7 days of reaction.

Ten days after the reaction, we observe the appearance of absorption spectra of nanoparticles and for all reaction times. The UV-vis data in Figure 4 shows a distinct peak at 416 nm is observed at all times of reaction (5-30) min. The figure also shows that the peaks at all times are identical with each other and similar to the absorption spectra in Figure 1. This indicates the formation of silver nanoparticles at all reaction times, and this is consistent with the results of Song and Kim, 2009; Verma and Mehata, 2016 and Sithara *et al.,* 2017.

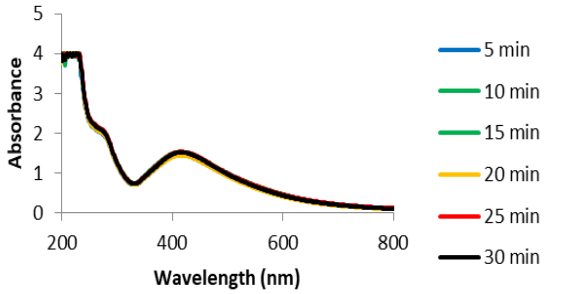

Figure 4. Absorption spectra of AgNPs at various reaction time intervals of *M. fragrance* hot aqueous extract and 10^{-3}M $AgNO_3$ at 60 °C after 15 days of reaction.

From the UV-VIS analysis, in Figure 5 that showed an increase in the absorbance with time indicating an increase in the forming of AgNPs a broad-spectrum bell-shaped curve was obtained in the range 350-550 nm. A Distinct peak at 416 nm showed a sharp surface plasmon resonance (SPR), which was specified for silver nanoparticles (Mahendran and Kumari, 2016; Owaid *et al.,* 2018 and Pirtarighat *et al.,* 2019).

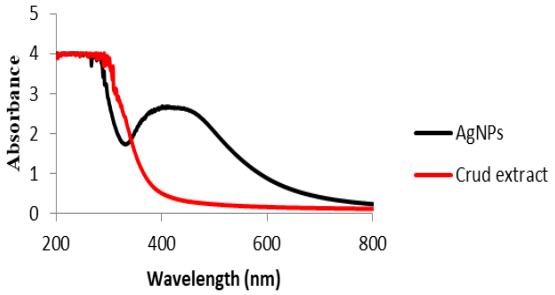

Figure 5 . Absorption spectra of silver nanoparticales and *M. fragrance* hot aqueous extract.

The effect of using different plant extraction methods in AgNPs synthesis

The results in figure 6 show increasing synthesis rate of AgNPs by using the hot aqueous extract of *M. fragrance*, then the boiling and cold plant extract. The extraction method may be the reason for this behavior which is due to the ionization of secondary compounds existing in aqueous extract and responsible for the reduction of silver salt to silver ions as a reducing agent, specially the essential oil and phenolic compounds found in nutmeg in large quantities (Raphael *et al.,* 2010; Enabulele *et al.,* 2014).

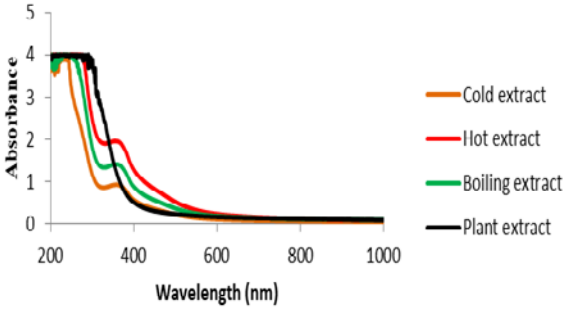

Figure 6 . Absorption spectra of AgNPs obtained from different aqueous extract methods for *M. fragrance* seeds with 10^{-3}M $AgNO_3$.

It is observed (Figure 6) that nanoparticles are formed clearly by using the three different aqueous extracts of *M. fragrance* seeds after interaction with silver salt (Sharma *et al.,* 2014; Verma and Mehata, 2016).

The effect of using different temperatures on AgNPs synthesis

The reaction mixture was exposed to different temperatures varying from 40 ºC to 70 ºC. The varying temperatures with the variation in absorbance of AgNPs synthesis were observed (Figure 7) indicating that temperature has altered the rate of AgNPs reaction. The highest rate of spectra value was at 60 ºC then 40 ºC but the lowest rate of spectra was at 50 ºC and 70 ºC.

The optimum temperature for AgNPs formation was recorded at 60 ºC, indicating that small Ag nanoparticles are formed at this temperature (Zia *et al*., 2016). As the temperature of reaction increased, both the rate of synthesis and the final conversion of silver nanoparticles increased (Song and Kim, 2009 and Ibrahim, 2015).

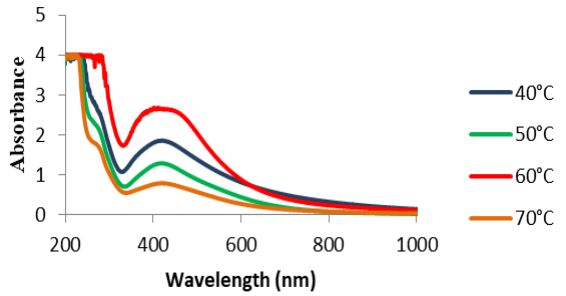

Figure 7. Absorption spectra of AgNPs prepared at different reaction temperatures of 40, 50, 60 and 70 ºC.

The effect of using different plant weights on AgNPs synthesis

For complete reduction of Ag ions to silver nanoparticles, three different weights of *M. fragrance* seeds were used to prepare the hot aqueous extract then mixed separately with a fixed volume of 10^{-3}M $AgNO_3$. The outcome in Figure 8 shows that at high concentration of 10 g / 100 mL of hot extract led to synthesizing more silver nanoparticles better than 6 and 8 g / 100 mL (Heydari and Rashidipour, 2015), which indicates there is a direct interconnection between the extract concentration and the synthesis of silver nanoparticles.

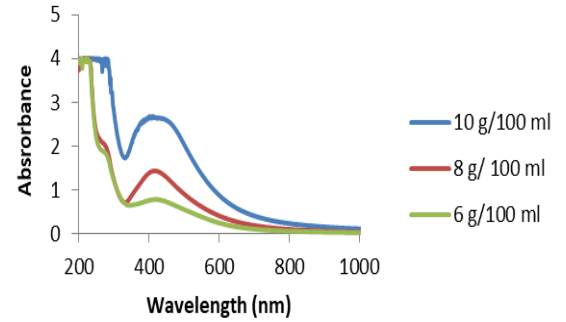

Figure 8. Absorption spectra of AgNPs at different weights of *M. fragrance* seeds extracts and 10^{-3}M $AgNO_3$.

The increase in extract concentration leads to rising in the rate of secondary compounds and thus increasing the reduction of silver salt to silver ions.

The effect of using different volumes of aqueous hot broth on AgNPs synthesis

Another important variable that determines the optimal volume of the extract reacts with silver salt to produce nanoparticles. Figure 9 shows the UV-visible spectra of nanoparticales obtained from the reaction of three different quantums of *M. fragrance* seeds hot extract and 10^{-3}M $AgNO_3$.

When increasing the volume of seed extract to silver nitrate to the ratio 45:5, 25:25 mL, no absorption band was observed. While decreasing the volume in the reaction mixture to 5:45 mL, the absorption was observed at 416 nm, indicating the enhancement in the synthesis of silver nanoparticles (Heydari and Rashidipour, 2015).

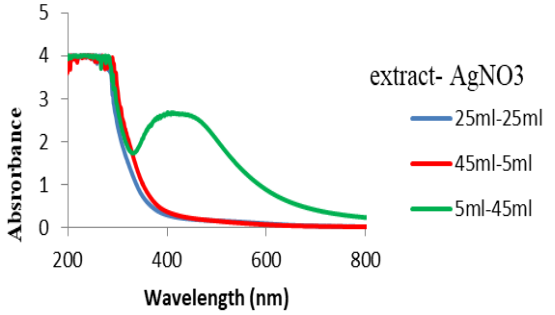

Figure 9. Absorption spectra of AgNPs at various volumes of *M. fragrance* hot aqueous extracts and 10^{-3}M $AgNO_3$ (25:25, 45:5, 5:45) mL.

2.7.2. FTIR spectroscopy analysis

FTIR measurement was carried out to identify various functional groups in *M. fragrans* seed extract and predicting their role in the synthesis of Ag NPs. The FTIR spectra of nutmeg extract and AgNps is shown in Figure 10 (a), (b) and (c) showing strong and broad range peak at 3441 cm^{-1}, that assigned for OH stretching in alcohols and phenolic compounds (Sharma *et al*., 2014 and Sivaprakash *et al*., 2019).

Shifting to the lower frequency 3442 cm^{-1} and 3439 cm^{-1} for silver nanoparticles at 12 and 16 min respectively may indicate the involvement of the O-H functional group in the synthesis of nanoparticles. The absorption peak at 1627 cm^{-1} could be assigned to the υ C=N stretching in the amide group in the seeds extract. The shift in this peak from 1627 cm^{-1} to 1635 cm^{-1} and 1637 cm^{-1} of AgNps that synthesized at 12 and 16 min respectively indicates the possible involvement of amide group in *M. fragrans* seed extract in nanoparticle synthesis.

The peak at 1066 cm^{-1} may attributed to the C-O group in crude seeds extract which shifted to 1062 cm^{-1} and 1060 cm^{-1} in silver nanoparticles at 12 and 16 min respectively after the reduction of silver. The FTIR results displayed that the biological molecules performed dual functions of synthesis and stabilization of AgNPs in the aqueous broth (Erjaee *et al.,* 2017; Khan *et al.,* 2018).

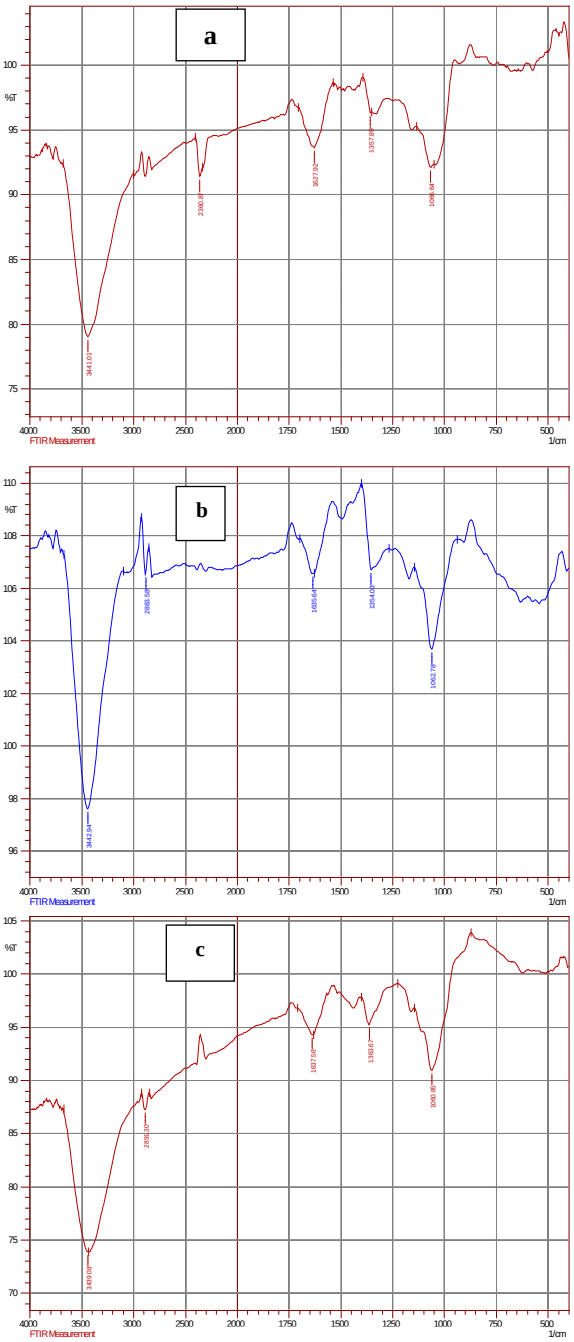

Figure 10.FTIR spectra of (a) *M. fragrans* aqueous extract (b) AgNPs in 12 min (c) AgNPs in 16 min.

2.7.3. Atomic Force Microscopy (AFM) analysis

AFM analysis is used to study the surface morphology of nanoparticles (Shkryl *et al.,* 2018). AFM images can determine the size, conglomerate, and surface roughness of silver nanoparticles (Majeed *et al.,* 2015 and Fatema *et al.,* 2019). The circumstances of an experiment are highly influence nanoparticles distribution and differences in size, and capability for aggregation.

Many shapes of ANPs appeared after synthesis by using the green method, like spheres, plates, rods and needles. The form of silver nanoparticles depends on the kind of plant and the concentration of plant broth, the rate of adding the plant extract and silver salt to the reaction, time of reaction, temperature, pH, etc.(Fatema *et al.,* 2019).

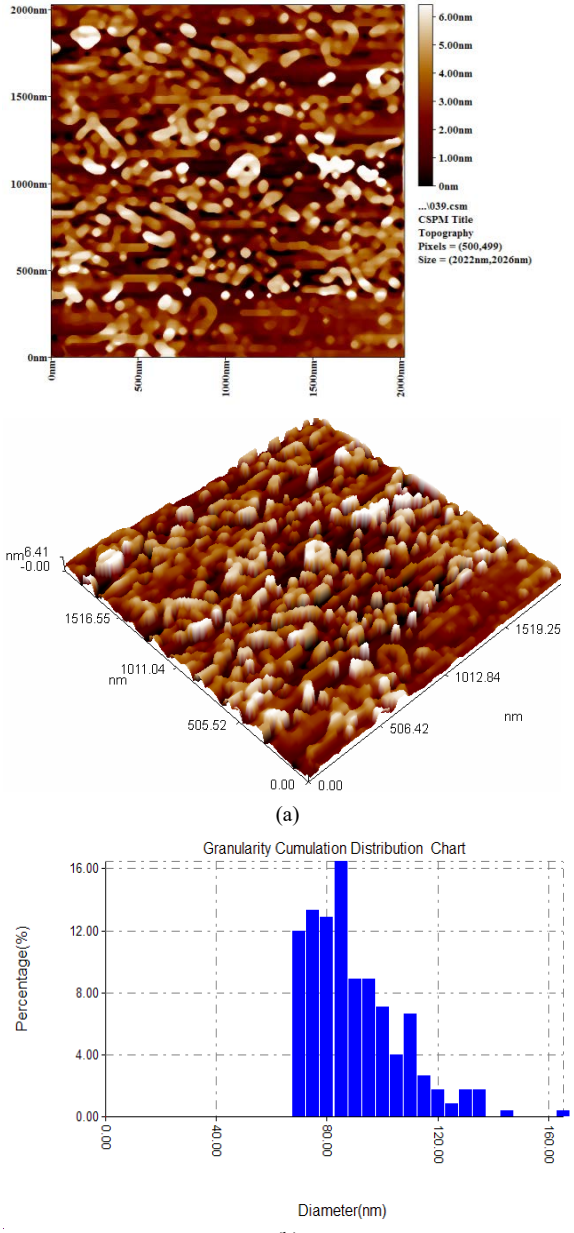

Figure 11. AFM topography for Ag nanoparticles **(a)** 2D & 3D AFM images of AgNPs that synthesized at 60 ºC after 12 min of reaction. **(b)** Granularity distribution of AgNPs that synthesized at 60 ºC after 12 min of reaction.

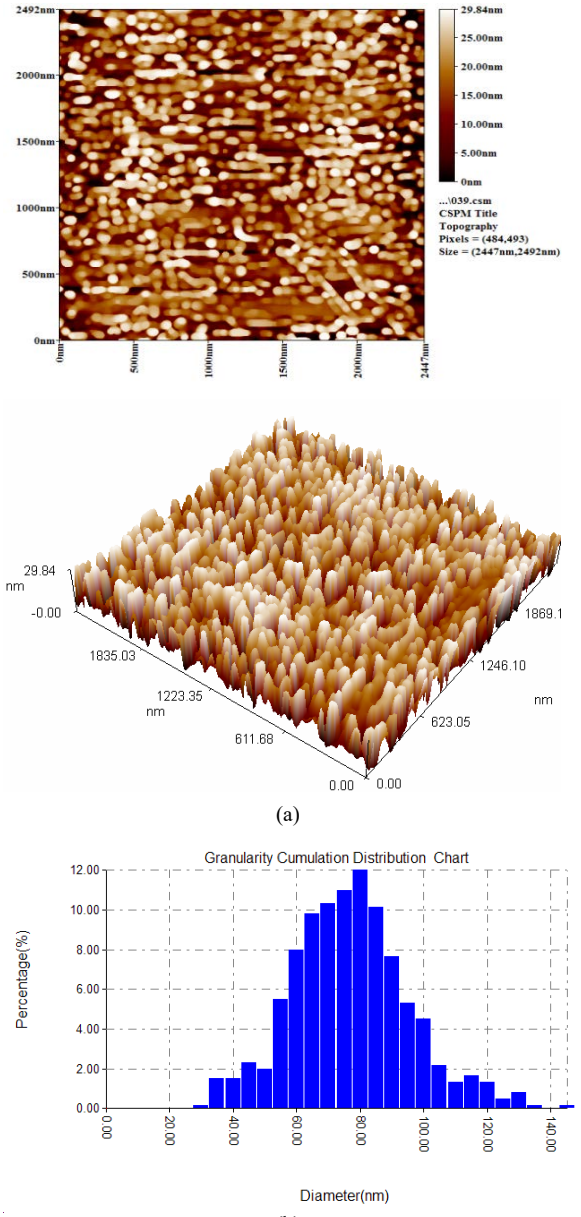

Figure 12. AFM topography for Ag nanoparticles **(a)** 2D & 3D AFM images of AgNPs that synthesized at 60 ºC after 16 min of reaction. **(b)** Granularity distribution of AgNPs that synthesized at 60 ºC after 16 min of reaction.

AFM images in Figure 11 (a) and 12 (a) show that Ag particles are in nanometer- size and granular shape. The size of silver nanoparticles were 87.71 nm and 74.55 nm for the samples taken after 12 min and 16 min of reaction as shown in Figure 11 (b) and 12 (b) respectively. The grain size rate decreasing with increasing the reaction time by increasing the silver salt reduction due to the ionization of secondary compounds as stated by AL-Azawi *et al.,* (2018).

2.8. The biological activity results

2.8.1. Antimicrobial activity of Ag NPs samples

Antibacterial activity of AgNPs samples a (12) and b (16) was studied against gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*) and gram positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*). The results show that the size of the nanoparticles and the kinds of bacteria under consideration have a great effect on the inhibition zone size. Figure 13 a shows that AgNPs sample (12) at the concentrations 100, 75, 50 and 25% caused the highest inhibition effect against *S. epidermidis* bacteria, and these results are compatible with the results of Netala, *et al.,* (2014) while the lowest inhibition effect for silver nanoparticles in all concentrations was against *S. aureus*. Both *E. coli* and *K. pneumoniae* bacteria achieved almost the same inhibition zone against using the nanoparticles in all its concentrations. Figure 13 b displays AgNPs sample 16 at 100% concentration exhibiting the highest inhibition effect against *E. coli* bacteria, and this is consistent with the results of Paul and Sinha, (2014). While *S. epidermidis* bacteria showed the highest inhibition zone when treated with silver nanoparticles at the concentrations 75, 50, and 25%, then *E. coli* bacteria at the same concentrations. While *K. pneumoniae* and *S. aureus* resulted in the same inhibition effect when tested in the presence of AgNPs sample 16 in all the concentrations.

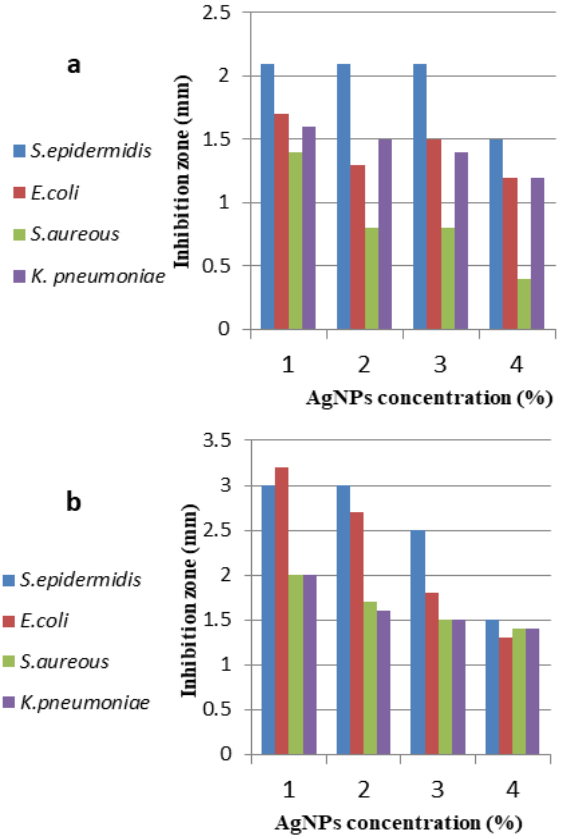

Figure 13. The effect of Ag NPs samples a (12) and b (16) on biological activity of pathogenic bacteria by using different concentrations (1) 100%, (2) 75%, (3) 50%, (4) 25% from silver nanoparticles.

2.8.2. Estimation of AgNPs effect on the biofilm formation inhibition by pathogenic bacteria

The influence of Ag nanoparticles on biofilm formation inhibition was clear for gram negative and positive pathogenic bacteria. The results confirm that there was an inverse relationship between the concentration of nanomaterials and the inhibitory effect of biofilm formation.

The lower the silver concentration, the more inhibiting effect on bacteria biofilm formation. The results also

proved that the lowest size of Ag nanoparticles sample (b) 16 (74.55) nm had a better inhibition effect on all bacterial strains than Ag nanoparticles sample (a) 12 (87.71) nm. It was observed that the highest effect of the biofilm inhibition for both nanoparticle samples 12 and 16 belonged to *K. pneumoniae* as shown in Figure 14.

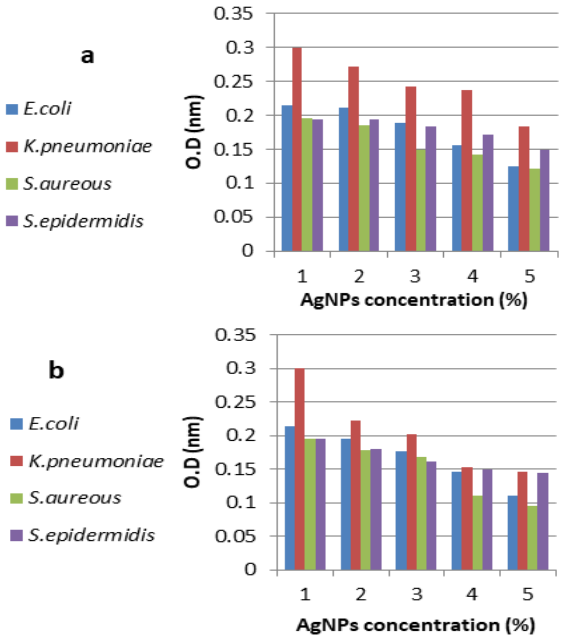

Figure 14. The effect of AgNPs samples a (12) and b (16) on inhibition of biofilm formation by pathogenic bacteria using different concentrations (1) (control) normal growth of pathogenic bacteria without AgNPs (2) 100%, (3) 75%, (4) 50%, (5) 25% from silver nanoparticles.

Both *E. coli* and *S. aureus* exhibited a convergent inhibition effect, while *S. epidermidis* bacteria showed the lowest inhibition effect compared with the control. AgNPs at the concentration 25% achieved the highest inhibition effect to biofilm formation among all the pathogenic bacteria, while the concentration 100% showed the lowest inhibition effect to biofilm formation among all pathogenic bacteria.

The best inhibiting biofilm effect of *K. pneumoniae* bacteria by using AgNPs was at the concentrations 25, 50, 75 and 100% respectively compared with the control while *S. epidermidis* showed less inhibition effect to biofilm formation at all the concentrations of AgNPs. The effect of AgNPs on Gram negative bacteria was better than the effect on Gram-positive bacteria. The mechanism of AgNPs activity occurs due to the correlation of AgNPs to the bacteria cell wall and then generating the free radicals (Pirtarighat *et al.,* 2019). Silver nanoparticles bind to the cell membrane, forming pores that increase membrane permeability and cause cell death (Netala *et al.,* 2014; Verma and Mehata, 2016 and Pirtarighat *et al.,* 2019).

2.8.3. Estimation the effect of AgNPs on plasmid curing of pathogenic bacteria

The AgNPs samples (12 and 16) displayed a clear inhibition effect on both Gram positive and negative bacteria as antiplasmid DNA activity shown in Figure 15.

S. aureus bacteria exhibited the highest inhibition effect for the entire concentrations (0.5, 1, 3 and 6) % for both samples 12 and 16 of silver nanoparticles compared with the control. *E. coli* bacteria followed the same behavior after being treated with the AgNPs samples 12 and 16 in all concentrations except 0.5% from sample 12 which displayed less effect as anti plasmid.

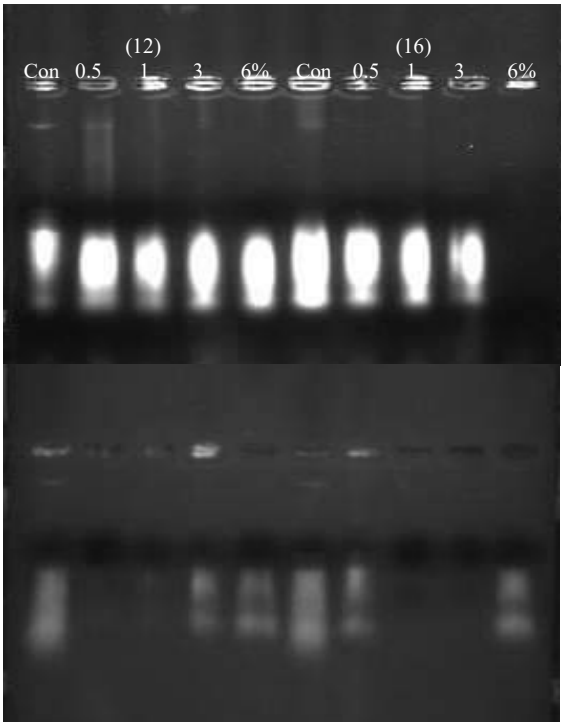

Figure 15. Agarose gel electrophoresis profile of plasmid DNA for *E. coli* and *S. aureus* bacteria after being treated with different concentrations (0.5, 1, 3 and 6) % from 12 and 16 samples of AgNPS, left: *E. coli* bacteria, right: *S. aureus* bacteria.

The results also proved that the lowest size of Ag nanoparticles sample (b) 16 (74.55) nm caused a better inhibitory effect as antiplasmid on pathogenic bacteria strains than Ag nanoparticles sample (a) 12 (87.71) nm.

3. Conclusion

The green synthesis of nanoparticles by using a green reduction agent is an ecofriendly, cheap, and effective method. In this study, AgNPs nanoparticles were synthesized by using the hot aqueous extract of *M. fragrance*. The chromatic change in the solution confirmed the formation of silver nanoparticles.

UV–vis spectra displayed a special peak for silver at 416 nm which was conformable to the surface plasmon peak of Ag nanoparticles; in addition, formation of silver nanoparticles occurred at all reaction times. The use of hot aqueous extract of *M. fragrance* increased the synthesis rate of Ag nanoparticles, especially after using 10 g / 100 mL from plant seeds to prepare the hot aqueous extract. The convenient temperature for AgNPs formation was

60 ºC, which indicates that a small Ag nanoparticle was formed at this temperature. While the optimal volume of the extract that reacts with silver salt to produce nanoparticles was 5:45 ml.

The FTIR results showed that the biological molecules are forming dual functions of synthesis and stabilization of AgNPs in the aqueous broth. AFM images prove that Ag particles were nanometer-size and had a granular shape.

The grain size rate decreased with increasing the time of reaction, the size of silver nanoparticle was 74.55 nm for the sample taken after 16 min of the reaction. The Ag

nanoparticles in the samples 12 and 16 in all concentrations showed the highest inhibition effect as antibacterial against *S. epidermidis* bacteria.

The influence of Ag nanoparticles on biofilm formation inhibition showed that the lowest size of AgNPs sample 16 (74.55) nm caused a better inhibition effect on all bacterial strains, while the highest effect of the biofilm inhibition for both nanoparticle samples 12 and 16 belonged to *K. pneumoniae*. The results proved that the lowest size of Ag nanoparticles sample (b) 16 (74.55) nm had a better inhibitory effect as anti plasmid on *S. aureus* bacteria strains than Ag nanoparticles sample (a) 12 (87.71) nm.

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